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Intestinal health and microbiota in salmonids: the impact of probiotics under potentially stressful conditions

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Intestinal health and microbiota in salmonids: the impact of probiotics under potentially stressful conditions

by

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Copyright Statement

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Author's declaration

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Intestinal health and microbiota in salmonids: the impact of probiotics under potentially stressful conditions

Alexander Jaramillo-Torres

The intestine and associated bacterial microbiota have a central role the physiology and homoeostasis of the host. The understanding of how farming conditions affect the intestine and associated microbiota of fish is the high importance to counteract the potential threats to health and welfare. Thus, this thesis aims to understand the role of stressful husbandry conditions on the intestine and associated microbiota of rainbow trout and Atlantic salmon. Within this context, the role of *Pediococcus acidilactici* as health promoter was also investigated

Chapter 3 investigated the replacement of fishmeal by different plant protein ingredients in rainbow trout. The results of this chapter revealed that the effect of *P. acidilactici* on the microbiota of distal intestine in rainbow trout was dependent on the ingredients of the diet. The results also showed that the FM substitution induced major changes in the intestinal microbiota. Moreover, the modulation induced by plant-based diets on the microbiota varied according to the ingredients used.

Chapter 4 studied the effect of dietary oxytetracycline in the distal intestinal microbiota of rainbow trout and the role of *P. acidilactici* to ameliorate the impact of antibiotic therapy. Experimental groups fed the diets with oxytetracycline had substantial changes in the distal intestinal microbiota including a decrease in the bacterial diversity. *P. acidilactici* did not ameliorate the effect of antibiotic therapy in the intestinal microbiota.

Chapter 5 used Atlantic salmon during smoltification to study the changes in the microbiota of distal intestine and the role of *P. acidilactici* to promote intestinal health. The results showed that bacterial communities in the mucosa differed from the digesta. Seawater transfer and *P. acidilactici* had significant changes in the intestinal microbiota of both mucosa and digesta. However, the modulatory effect of both factors evaluated was larger in the mucosa-associated microbiota than in the digesta-associated microbiota. Furthermore, *P. acidilactici* induced a significant increase in antiviral-related genes.

Chapter 6 investigated the replacement of fish oil by rapeseed oil alone or combined with *P. acidilactici* on the intestinal health and microbiota of two intestinal regions in Atlantic salmon. Replacement of fish oil by rapeseed oil alone or in combination with *P. acidilactici* supplementation did not induce major changes in the intestinal health and microbiota. The bacterial communities found were significantly different between the pyloric caeca and mid-intestine.

In conclusion, this thesis contributes to new knowledge regarding the effect of dietary supplementation of *P. acidilactici* and the impact of different potential challenging factors in the health and intestinal microbiota of farmed salmonid species.

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List of abbreviations

ANOVA	Analysis of variance
AU	Arbitrary units
cDNA	Complementary deoxyribonucleic acid
CFU	Colony forming unit
DHA	Docosahexaenoic acid
EFSA	European Food Safety Authority
EPA	Eicosapentaenoic acid
FAO	Food and Agriculture Organization of the United Nations
FCR	Feed conversion ratio
FM	Fishmeal
FO	Fish oil
GALT	Gut-associated lymphoid tissue
GF	Germ-free
GI	Gastro-intestinal
HSI	Hepatosomatic index
HSP	Heat shock protein
HTS	High-throughput sequencing
IEL	Intra epithelial leucocyte
LAB	Lactic acid bacteria
LEfSe	Linear discriminant analysis effect size
MAMPs	Microbe-associated molecular patterns
MS222	Tricaine methane sulphonate
OTU	Operational taxonomic unit
PBS	Phosphate buffered saline
Pcna	Proliferation cell nuclear antigen
PCoA	Principal coordinates analysis
PCR	Polymerase chain reaction
PD	Phylogenetic diversity
QIIME	Quantitative insights into microbial ecology
PRR	Pattern recognition receptor
RNA	Ribonucleic acid
RO	Rapeseed oil
RT-PCR	Real time polymerase chain reaction
SBM	Soybean meal
SD	Standard deviation
SGR	Specific growth rate
SPC	Soy protein concentrate
WG	Weight gain

CHAPTER 1. INTRODUCTION

1.1. Aquaculture outlook

According to the United Nations, the world population for 2015 was 7.3 billion. Projections suggest that this value will rise to 8.5 billion by 2030 and 9.7 billion in 2050 . (United Nations et al., 2015). This rapid growth will bring central challenges regarding food production and food security. Animal production sector, as well as other agriculture segments, are responsible for meeting this challenge, finding a balance between increasing the production efficiently without detriment to fish welfare and environment in order to remain sustainable in the long term.

Fish production (combining capture wild fisheries and aquaculture) has contributed to meet the animal protein demand for the growing human population, being the largest source of animal protein in the world above other sources such as swine, poultry and cattle industries (Figure 1.1a). Fish provide an important portion of the animal protein intake for the global population, according to The Food and Agriculture Organization of the United Nations (FAO), fish accounted for 20% of the average per capita intake of animal protein for 3.1 billion people, with substantial variations between regions (FAO, 2016). In this context, the role of aquaculture in providing a protein source for human consumption has been remarkable considering that fish production from wild fisheries has remained relatively stable since 1990 and fish consumption per capita has increased from approximately 9.9 kg in the 1996 to 19.1 kg in 2012 (Lem et al., 2014). To meet this demand, fish production has increased from 20 million tonnes in 1950 to 156.2 million tonnes in 2012 (capture fisheries and aquaculture), becoming the world's fastest growing food production sector (Figure 1.1a). Currently, aquaculture provides almost 50% of all fish for human consumption; this value is expected to increase 62% by 2030 (FAO, 2014).

a) b)

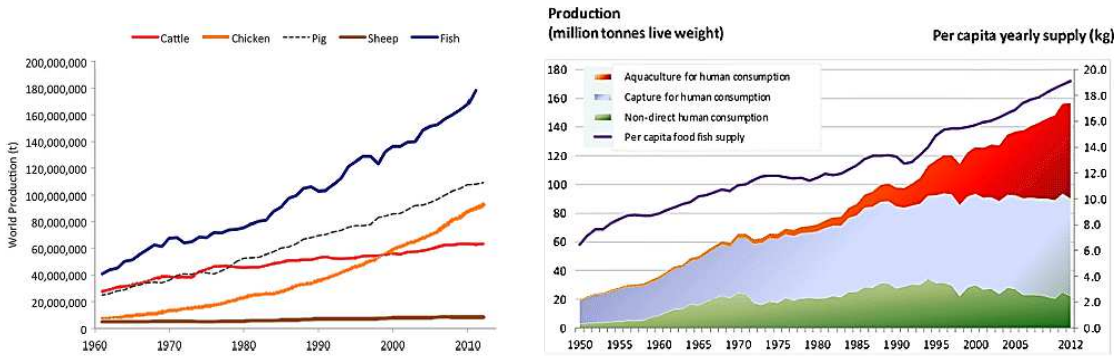


Figure 1.1. World animal production. a) World production of animal protein (1960-2010); b) Fish production from aquaculture and capture for human consumption. Adapted from Béné et al. (2015).

1.1.1. Salmonid aquaculture

Salmonid aquaculture is one of the leading production systems in the aquaculture industry and an appreciated market regarding the quality of the protein provided for human consumption. In the last decades, production of salmonids has expanded in Northern Europe and North and South America (Figure 1.2.). The most important salmonid species are rainbow trout (*Oncorhynchus mykiss*) and Atlantic salmon (*Salmo salar*). Rainbow trout is mainly cultured in Chile, Iran, Turkey and Norway. Meanwhile, Atlantic salmon production is principally based in Norway, Chile, Scotland, Faroe Islands and Canada (FAO-FIGIS). Salmonid aquaculture is characterised for being highly industrialised, and their production is mainly carried out intensively (Asche and Bjorndal, 2011). The success of this industry has been based on investment in technology and innovation to improve the productivity and to provide economic development for coastal communities (Asche and Bjorndal, 2011).

Atlantic salmon is the most commercially important salmonid species, and it is, together with shrimps, the most intensively farmed and traded species in the aquaculture industry (FAO, 2014). Regarding consumption, Atlantic salmon is one of

the top five of the most important products in seafood markets, and it is considered as a high-value species regarding its human nutritional value. As other oily fish, salmon is an excellent source of omega-3 (n-3) fatty acids, which are widely associated with health benefits for humans. Based on this, the European Food Safety Authority (EFSA) recommends a daily consumption of 250 mg EPA (eicosapentaenoic acid) and DHA (Docosahexaenoic acid) through oily fish (EFSA Panel on Dietetic Products, 2010).

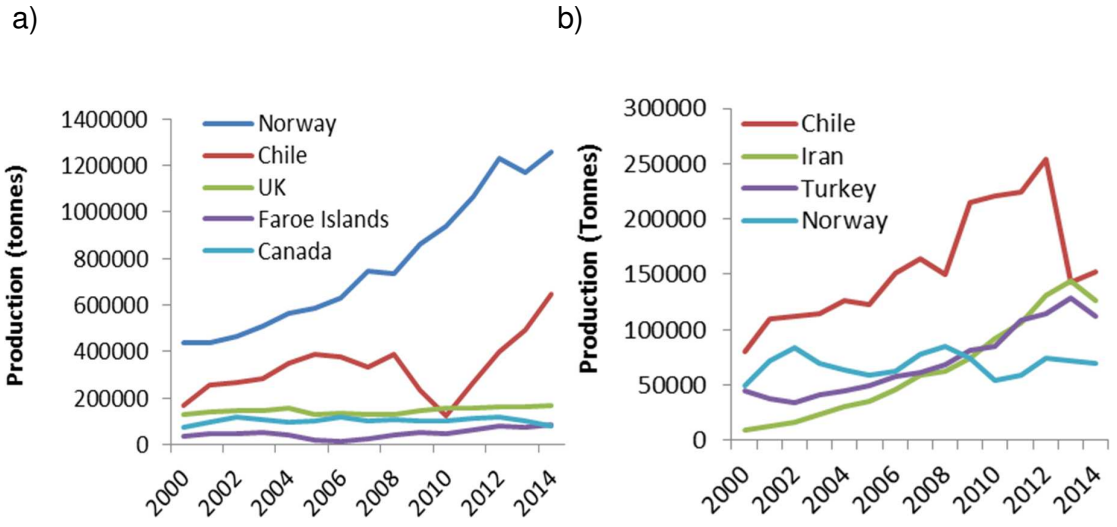


Figure 1.2. Main salmonid producers in the world between 2000-2014. a) Atlantic salmon; b) rainbow trout. Data from FAO-FIGIS.

1.1.2. Challenges for salmonid industry

Despite the success that the salmonid industry has enjoyed, the industry is currently facing several challenges that have restricted its expansion and sustainability (Torrissen et al., 2011). Some of the main issues affecting the industry are related to the supply of raw materials for feed production and the occurrence of infectious diseases affecting the farmed fish. As salmonids are carnivorous fish, their production

is highly dependent on fishmeal and fish oil for the formulation of diets (Naylor et al., 2009). Due to the variable market prices of fishmeal and fish oil and depletion of wild fisheries, the use of these resources as a unique source of proteins and lipids is re-evaluated. Proteins and lipids from plants have been studied and used as alternatives to fishmeal and fish oil in the diet of salmonids (reviewed by Oliva-Teles et al. 2015). However, different studies have confirmed that the presence of antinutritional factors in plant ingredients has an impact on fish health, particularly in carnivorous species, restricting the level of inclusion of these sources to replace marine ingredients for salmonid diet (reviewed by Krogdahl et al. 2010). Furthermore, a high replacement of marine ingredients in diets for salmon may affect its nutritional value affecting the quality of the final product, especially the profile of omega-3 (n-3) fatty acids (Sprague et al., 2016).

On the other hand, salmonids under farm conditions are exposed to a wide variety of potential health threats including parasites, bacteria and virus infections that may have severe consequences for the health and welfare of the fish as well as important social and economic impact. The outbreak of a viral infection, namely infectious salmon anaemia in the Chilean Atlantic salmon production in 2007, it is an example of the devastating social and economic consequences of infectious diseases in the salmonid industry. It was estimated that during the outbreak at least 15,000 jobs were lost and the direct economic impact was approximate US$ 2 billion from 2007 to 2009 (Mardones et al., 2011). Infectious pancreatic necrosis and pancreas disease are also viral diseases frequently reported to cause important losses for the industry. Additionally, parasites such as sea lice and Amoebic Gill Disease have been major constraints in the salmon industry (Costello, 2009; Shinn et al., 2015). Rainbow trout are also susceptible to different infectious diseases including those caused by bacteria

Flavobacterium psychrophilum and *Piscirickettsia salmonis* but also viral diseases such as viral haemorrhagic septicaemia and infectious haematopoietic necrosis (Dale et al., 2009; Yu et al., 2016). Control of pathogens in salmonids has been traditionally based on the use of immunisation and chemotherapeutic. Nonetheless, some of these approaches have limitations including lack of vaccines for some important viral and bacterial pathogens and antibiotic and pesticide resistance for bacteria and parasite agents respectively. Novel strategies are necessary to tackle the current sanitary problems in salmonids aquaculture. Some suggested strategies are based on holistic approaches including improvement of the farming conditions and fish welfare, manipulation of water and gut microbiota, use of functional feeds (De Schryver et al., 2012), improvement of genetic resistance through selective breeding (Moen, 2010) and increase of efforts to produce new vaccines and novel chemotherapeutic agents.

1.2. The gastrointestinal tract of salmonids

1.2.1. Organisation and function

The normal structure and function of the gastro-intestinal (GI) tract in fishes is similar to other vertebrates with special adaptations that reflect their feeding habits, life cycle, and functional demands. Due to the high phylogenetic diversity in the fish group, only the GI tract of some species, especially fish with relevant importance in aquaculture, have been studied more extensively. The GI tract is essentially a tube that starts in the oral cavity and extends along the fish body until the anus, which is constituted by different regions with important functional and morphological differences.

Even though salmonid species, especially Atlantic salmon and rainbow trout, are key species in aquaculture, relatively few studies have been published comprehensively

describing the morphological features of different regions of the GI tract macro and microscopically. In salmonids, the GI tract can be divided into four main regions; oesophagus, stomach, pyloric caeca, and intestine according to macroscopic differences (Sanden et al., 2005). To date, there has been little agreement on the nomenclature used to describe the different anatomical regions of the GI tract of salmonids. Particularly, the number of divisions and names for the intestine is a matter of some confusion and debate. For example, Løkka and Koppang (2016) used zebrafish nomenclature despite the fact that this species does not share the same anatomic regions to salmonids. Thus, these authors suggest to divide the intestine into five parts; pyloric caeca, first segment of the mid-intestine, second segment of the mid-intestine and posterior segment. In contrast, other authors subdivide the intestine into three parts; proximal, mid, and distal intestine (Krogdahl et al., 1999). In this thesis, the nomenclature to describe the intestine of the salmonids used during the experiments will be based on a three-part subdivision as described in Figure 1.3.

Generally, in fish, the GI tract is heavily folded and in contrast with mammals does not have villi or crypts. In salmonids, the proximal intestine is characterised by the presence of blind-ended ducts named pyloric caeca. The number of these structures typically ranges between 50-62 (Peruzzi et al., 2015). The function of pyloric caeca is not entirely clear, but it is hypothesised that they are surface-increasing structures involved in enzymatic breakdown and absorption of fatty acids (Denstadli et al., 2004) as well as glucose, amino acids, and dipeptides (Bakke-McKellep et al., 2000). The mid-intestine has similar histological appearance to the proximal intestine and has been recognised, together with the proximal intestine as the main regions for nutrient absorption. The distal intestine has different histological organisation to the proximal and mid-intestine. Complex circular folds are abundant and larger in the distal intestine

with aggregates of goblet cells at the apical end; mucus production is reported to be higher than in more proximal segments (Løkka et al., 2013). A distinctive histological feature of this region is the presence of enterocytes with supranuclear vacuoles which are associated with uptake of macromolecules and antigen-sample from the lumen (Urán et al., 2008; Løkka and Koppang, 2016). The distal intestine in salmonids has been considered to have major immunological functions (Petrie and Ellis, 2006), higher transcript level of immunological genes has been reported in this segment in comparison with the most proximal parts of the intestine (Løkka et al., 2014).

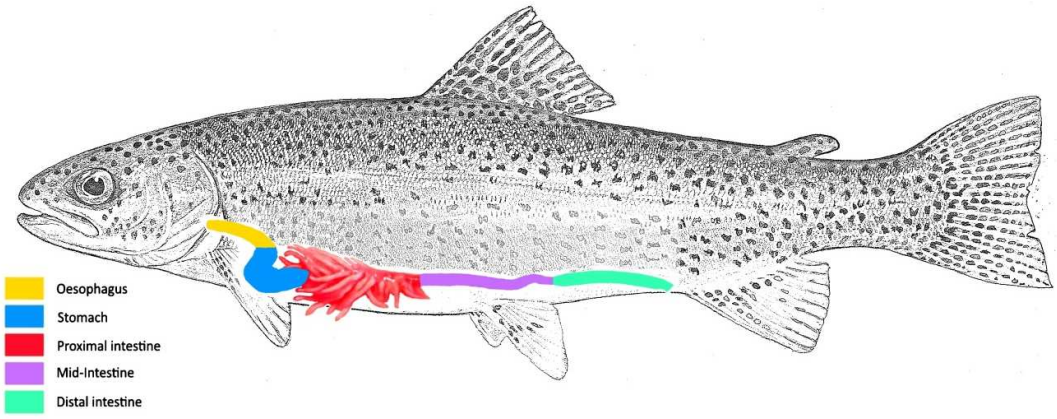


Figure 1.3. Gastrointestinal tract of rainbow trout showing oesophagus, stomach, and intestine with their three subdivisions: a) proximal intestine: the region where presence of pyloric caeca is observed, from pyloric sphincter to the last pyloric cecum is connected; b) mid-intestine: from the portion immediately after the most distal pyloric caeca to the beginning of the distal intestine which is clearly distinctive from mid-intestine due to the presence of annular rings, darker mucosa and increased diameter; c) distal intestine: from the border of mid-intestine until the most distal part of the intestine.

1.2.2. The intestinal barrier

Traditionally, the most widely studied function of the intestine is related with digestive physiology. However, the intestine in fish, as in other vertebrates, is multifunctional and includes roles in metabolism, osmoregulation, immunity and respiration in some particular species (Wilson and Castro, 2010). The mucosa of the GI tract represents a vast surface area in immediate contact with the external environment. The GI

mucosa has a major role in maintaining the homoeostasis by keeping out the intestinal microbes and other undesirable substances such as toxins. Therefore, an intact intestinal barrier is critical for the fish health. The intestine is constituted by specialised groups of cells such enterocytes, goblet cells, enteroendocrine cells and a wide variety of immune cells (Salinas and Parra, 2015). These cells and their products constitute key players of the so-called intestinal barrier that protect the host against potential pathogens and foreign antigens from the luminal environment. The intestinal barrier also has an important role in controlling the intestinal permeability.

1.2.2.1. Intestinal mucus

The intestinal mucus plays a central role as part of the intestinal barrier, forming a chemical and physical protection for the underlying epithelium. The mucus is produced by the goblet cells, which are abundant in the intestine and localised between enterocytes. The main proteins in the mucus are the mucins, high molecular weight glycoproteins that can bind to the water conferring the typical gel-like properties of the mucus (viscosity, slipperiness, and stickiness).

Recent evidence in mammals suggests a strong interaction between the intestinal microbiota and mucus properties. A comparison between conventionally raised and germ-free (GF) mice indicated important differences in the mucus properties. The mucus from GF mice was easier to penetrate by bacterial-size beads compared to mucus from conventional raise mice, i.e. mice with normal intestinal microbiota. Moreover, the mucus from GF mice that were colonised with the same microbiota as conventionally raised mice took six weeks to become impenetrable (Johansson et al., 2015). This study suggests that the intestinal microbiota may modulate mucus properties. On the other hand, the mucus contributes to control of the microbiota in the intestine. As a result of the constant renewal of the mucus layer, the microorganisms

in the lumen are removed. Nonetheless, the mucus also offers a nutrient-rich microenvironment mainly composed of polysaccharides, which supports the colonisation of commensal bacteria (Fabich et al., 2008).

Even though the main components of the intestinal mucus are the mucins, other active components help to protect the intestine. Two components of the mucus that have been described as necessary for the immunity of the intestine are antimicrobial molecules and immunoglobulins. Some of the antimicrobial molecules detected in the fish gut mucus are lysozyme, complement components, lectins antimicrobial peptides and cytokines (reviewed by Salinas and Parra 2015) . The immunoglobulins detected in the mucus of fish are IgM and IgT. Immunoglobulin T has been found to be able to coat a large percentage of luminal bacteria in the intestine in the same way that IgA plays this function in mammals, suggesting that this immunoglobulin has a key role in preventing the attachment and invasion of the intestinal epithelium (Zhang et al., 2010). All these molecules are thought to have an important influence in the protection of intestinal mucosa.

1.2.2.2. Intestinal epithelial barrier

Below the intestinal mucus, there is a barrier composed of a single layer of tightly connected epithelial cells that are specialised in absorbing nutrients such as proteins, fatty acids, and carbohydrates. Despite their role in nutrient absorption, epithelial cells have a sophisticated defence mechanism to respond to potential hazards. For example, epithelial cells are able to sense pathogens using highly conserved receptors and also to release antimicrobial molecules and cytokines to trigger a more complex response together with surrounding immune cells (Pastorelli et al., 2013).

The epithelial cells are connected by several families of intercellular proteins known as tight junctions. These proteins have been widely investigated in human and other mammals (Pastorelli et al., 2013). In salmonids, many of these proteins have also been identified (Reviewed by Sundell and Sundh 2012). Although the function of each of the proteins forming the tight junction in fish in not well understood, similar to other animals, the tight junctions have a central task in coordinating the permeability of the intestinal barrier. The interaction between all the tight junction proteins and membrane lipid composition of enterocytes including aquaporins control the permeability in the fish intestine (Engelund et al., 2013; Madsen et al., 2014). It is important to highlight that the different segments of the intestine differ in specific functions and may thus have different permeability as is the case in humans (Jutfelt, 2011).

1.2.2.3. Intestinal immune barrier

Fish were the first vertebrates to develop an adaptive immune system; however, in contrast with mammals, fish are poikilothermic animals. This high dependence on the surrounding temperature affects the body metabolism including the speed of the immune system to develop a response to a threat. Antibody production after vaccination in cold-water fish such as Atlantic salmon or rainbow trout is only detectable 4 to 6 weeks after immunisation, whereas, a warm-water species such as hybrid striped bass (*Morone saxatilis* × *Morone chrysops*) is able to develop detectable antibodies one week after vaccination (reviewed by Sommerset et al. 2015). Since the adaptive immune system in fish is considerably slower than the innate immune system in developing an appropriate defence response, it is suggested that the innate immune system in fish is more important for fish than in mammals for defending the host against pathogens.

The immunological response in the intestinal mucosa is mainly coordinated by gut-associated lymphoid tissue (GALT). The gut immune system in teleosts has important differences from that of mammals, especially the absence of Peyer's patches and mesenteric lymph nodes as well as in the immunoglobulins involved. As it was mentioned previously IgT is the immunoglobulin present in the intestinal mucus; this immunoglobulin is suggested to be specialised to mucosal immune responses in salmonids (Zhang et al., 2010).

The intestine is constantly exposed to a wide repertory of antigens from pathogenic and commensal microorganisms as well as food and self-antigens. Thus, it is a central role of the GALT, the immune surveillance of the intestine, to determine whether to initiate an immune or tolerance response after being in contact with a specific antigen (Kiron, 2012). After a disruption of the intestinal barrier or the presence of a potential pathogen, the intestine needs a balanced immune response; otherwise, an uncontrolled inflammatory response can take place. The GALT is responsible for preventing and neutralising potentially harmful microorganism or their antigens from reaching the systemic circulation. In order to recognise the pathogenic nature of the threat, the GALT and epithelial cells have conserved receptors, the so-called pattern recognition receptors (PRR), of which the most studied are the Toll-like receptors (TLR) which have been found in different fish species. The TLR are proteins with a pivotal role in the innate immune system. These proteins are able to sense conserved structures from microorganisms; the microbe-associated molecular patterns (MAMPs). MAMPs are small conserved motifs that are unique to microorganisms and essential for their physiology. Some of the MAMPs are part of the cell wall of bacteria (lipopolysaccharide and peptidoglycan) and fungi (β-glucan). Immune cells can express specific TLR for different MAMPs allowing the immune system to differentiate

between self and non-self antigens. However, it is important to highlight that MAMPs are not only present in pathogenic microorganisms but also present in commensal microorganisms inhabiting the intestine. Thus TLR and other PRRs are not able to differentiate between pathogenic and commensal microorganisms. Commensal microorganisms have crucial functions in the intestine and, under normal conditions, they keep a symbiotic relationship with the intestine. Thus, the GALT does not trigger an inflammatory but a tolerogenic response. The mechanisms behind the development of a tolerogenic response to the commensal microorganisms in the intestine by the immune system are poorly understood. Some theories suggest that in mammals tolerance to commensal microorganism is related to the tolerance to food antigens, which occurs in early life stages, and it is mediated by regulatory T cells (Gensollen et al., 2016).

A healthy intestine responds effectively to the potential challenges through a coordinated response between immune and nonimmune cells. The main immune cell populations found in the intestine are: a) lamina propria leukocytes, mainly macrophages, granulocytes, lymphocytes and plasma cells; b) intraepithelial lymphocytes (IEL), predominantly T cells and a few B cells. Other non-immune cells are also important in the intestinal immune response, principally, epithelial cells, goblet cells, and neuroendocrine cells.

1.3. Fish gut microbiota

The GI tract of human and other animals offers excellent conditions for the establishment of a diverse and complex community of microbes including viruses, yeasts, protists and bacteria (Romero et al., 2014). These microbial inhabitants of the gut have a close interaction with the host and are able to modulate different

physiological functions (Weinstock, 2012). The importance of gut microbiota has been extensively studied in humans and other endothermic animals (Grenham et al., 2011). Despite this, remarkable progress has been made in this field in the last 20 years. Several studies have reported that gut microbiota can modulate different physiological and nutritional aspects in fish (reviewed by (Ray et al., 2012; Lazado and Caipang, 2014b; Romero et al., 2014).

Before the application of molecular methods to characterise the gut microbiota in fish, the knowledge of the microbes associated with the gut was based on culture-dependent methods (Trust and Sparrow, 1974; Ringø et al., 1995). Culture-dependent methods to assess microbes rely on the ability of the method to grow the microorganism based on their nutritional and physiological requirements. Thus, only a minor percentage of the bacteria are capable of growth on common laboratory media (Spanggaard et al., 2000; Pond et al., 2006). Currently, it is widely accepted that this approach underestimates the microbial population present in the gut as it does not take into account the non-culturable microorganisms as well as those with strict growth conditions under laboratory conditions (reviewed by Zhou et al., 2014) Studies in salmonids have reported variable cultivable bacteria in the gut, 9% to 27% in trout (Navarrete et al., 2010), 3% in juvenile coho salmon (Romero and Navarrete, 2006) and less than 1% in juvenile Atlantic salmon (Navarrete et al., 2009). These studies highlight the importance of using culture-independent methodologies to have a more comprehensive overview of microbial communities in the fish gut.

Molecular techniques based on detection of bacterial DNA are becoming more popular to study the complex bacterial communities such as those found in the fish gut. The use of molecular methodologies such as the 16S rRNA gene clone libraries (Li et al., 2015), denaturing gradient gel electrophoresis (DGGE) (Merrifield et al., 2009),

temperature gradient gel electrophoresis (TGGE/TTGE) (Navarrete et al., 2010), hybridisation technique-fluorescence in situ hybridization (FISH) (Huber et al., 2004) and next generation sequencing (Desai et al., 2012; Gajardo et al., 2016b) have been used to assess the gut microbiota in fish. The application of molecular tools to study intestinal microbiology has facilitated the understanding of the complex microbial community resident in the fish gut. This new approach has also arisen as a powerful tool to understand how the microbial communities behave in the intestine under both natural and farm environments (Li et al., 2014; Zarkasi et al., 2014).

Traditionally, gut microbiota has been classified according to its ability to colonise the intestinal mucosa as autochthonous for the ones able to colonise the gut and transient bacteria or allochthonous for the microorganisms present in the luminal content or digesta. The concept of autochthony has been adopted from endothermic animals and traditionally used in aquatic organisms. In 1999 Ringø and Birkbeck (1999) suggested that autochthonous microorganisms should meet the following criteria:

a. Be harboured by healthy individuals.

b. Colonise early life stages and persist throughout life.

c. Be found in both free-living and hatchery-cultured fish.

d. Be able to grow anaerobically.

e. Be associated with the epithelial mucosa in the stomach, small intestine or large intestine.

Despite the importance that autochthonous microbiota could have on the host due to the close interaction of these microorganisms with the intestine, many of the recently

published studies using molecular approaches have been focused on investigating the allochthonous microbiota (Larsen et al., 2014; Li et al., 2014; Zarkasi et al., 2014).

To establish the “normal” gut microbial composition in fish has been a complex task as fishes have the highest species diversity in the group of vertebrates with more than 32,000 species distributed in a vast range of different aquatic environments. It is estimated that more than 300 species of finfish are farmed (reviewed by (Teletchea and Fontaine, 2014). Thus, the studies characterising the gut microbiota have been focused on species with high value for aquaculture industry such as tilapia, trout, salmon and carp and these with biomedical interest i.e. zebrafish (reviewed by (Romero et al., 2014). Even more, there is evidence that the gut microbiota could have high differences in the same species under different conditions of farming which has made difficult to establish the concept of a stable shared set of microorganisms commonly known as “core microbiota” of a determined species of fish.

1.3.1. Importance of gut microbiota in fish

To further characterise the microbiota communities established in the gut, valuable evidence has been published on the importance of these microbes in modulating physiological and nutritional aspects in fish (reviewed by (Perez et al., 2010; Ray et al., 2012; Gomez et al., 2013).

Despite a significant number of studies published in the last years on microbial communities in the gut of different fish species, the role that gut microbiota plays in fish nutrition is not completely understood. However, it is well known that many bacteria isolated from the gut of fishes produce exogenous enzymes with the capacity to digest different kinds of nutrients. Recently, Ray et al. (2012) performed

a comprehensive review of the studies investigating the enzymes produced by bacterial microbiota in fish gut. These authors reported a wide number of enzymes produced by fish gut microbiota. The production of enzymes such as amylase, cellulase, lipase, proteases, chitinase and phytase by fish microbiota, suggests an active function of the gut microbiota in the digestive process. Moreover, other studies have demonstrated that bacteria belonging to the gut microbiota produce vitamins (e.g., vitamin B12) and polyunsaturated fatty acids. However, the contribution of these bacteria to the fish nutrition has not been clarified (Nayak, 2010). Nonetheless, Sun et al. (2009) studied the differences in the gut microbiota of two groups of grouper (*Epinephelus coioides*) with fast or slow growth patterns. Even though the number of gut bacteria was similar between both groups, the presence of different bacterial species in each group suggests that the fast growing grouper had a more beneficial microbiota. Moreover, several authors have demonstrated that manipulation of the gut microbiota of fish using prebiotics and probiotics has a beneficial effect on the growth of different fish species (Avella et al., 2010a; Merrifield et al., 2010a; Ebrahimi et al., 2012).

Studies using gnotobiotic fish have provided relevant information about the role that the microbiota plays in the host. Rawls et al. (2004) determined that commensal bacteria modulate gene expression in the digestive tract in a GF zebrafish model. The same study evidenced that the intestine-associated microbiota stimulates intestinal epithelial cell proliferation. Moreover, other studies in zebrafish have reported the importance that microbiota has in the metabolism and absorption of fatty acid (Semova et al., 2012).

1.3.2. Effect of stress on intestine and associated microbiota

Farming conditions are unavoidably stressful for fish. For this reason, their impact cannot be denied on physiological functions which *in extremis* can cause, as a consequence, suboptimal production conditions represented in high mortality, susceptibility to diseases and low growth and reproduction performance (Pickering, 1993; Iwama, 1997). Therefore, the recognition of stress as an inherent part of finfish husbandry management and actions to mitigate their effects is a central challenge to improve the fish welfare and productivity of the aquaculture sector. Therefore, practices directed to mitigate negative stress conditions will be reflected in benefits for farmers, consumers and fish welfare (Conte, 2004).

Despite a large number of studies on the stress response in fish, the available information regarding stress effects on the GI tract is scarce. The first studies in this field were focused on describing the histopathological changes on the GI tract produced under different stress conditions such as transportation, catching and dominance hierarchies (Ringø et al., 2014). To the author's knowledge the first known study about the effect of stress on GI tract was carried out by (Peters, 1982); this author reported that social stress in European eel (*Anguilla anguilla* L.) caused an increase of mucus secretion and ultrastructural damage of gastric cells in the stomach. Another study determined that stress caused by catching and transport was associated with loss of goblet cells and columnar epithelial cells in the intestinal mucosa of carp (*Cyprinus carpio, Cyprinus carpio haematopterus*) (Szakolczai, 1997). Afterwards, Ringø et al. (1997) observed that individuals of Arctic charr (*Salvelinus alpinus* L.) with distinct hierarchy formation had changes in the diversity and total number of cultivable intestinal bacteria. These changes were attributed to stress

conditions caused by dominant individuals upon subordinate individuals. Unfortunately, this research did not use stress markers to differentiate the physiological response between dominant and subordinate individuals.

Other investigations evaluated the influence of acute stress on the GI tract of Atlantic salmon (Olsen et al., 2002), rainbow trout (Olsen et al., 2005) and Atlantic cod (*Gadus morhua* L.) (Olsen et al., 2008). These studies used a more comprehensive approach to evaluating the effect of stress, taking into account cortisol levels and ultrastructural changes in the intestine determined by electron microscopic examination, in comparison with previous research (Peters, 1982; Szakolczai, 1997; Ringø and Birkbeck, 1999).

Olsen *et al.* (2002) reported that acute stress conditions in Atlantic salmon induced substantial changes in the ultrastructure of enterocytes lining. This finding was also associated with damage of the intercellular junctional complexes. Furthermore, the intestinal microbiota was also affected by acute stress. The population level of cultivable adherent bacteria from hindgut tissue decreased, and an increase in the faeces was observed in comparison to non-stressed controls. Similar results in gut histology and intestinal microbiota were reported in the GI tract of rainbow trout affected by acute stress, in which an increase in the intestinal permeability was also noted (Olsen et al., 2005). These studies provide valuable evidence that stress has a significant influence on GI tract function affecting its structure and associated microbiota.

Significant variations regarding stress response and its effect on GI tract between fish species have been reported; this makes difficult to extrapolate results from another fish. Research carried out on Atlantic cod using similar experimental conditions as the

previously mentioned studies in salmonids (Olsen et al., 2002; Olsen et al., 2005), determined that acute stress not produce significant changes in the ultrastructure of GI tract. The latter could indicate that Atlantic cod is more resistant (tolerant) to the evaluated stress conditions in contrast to salmonid species (Olsen et al., 2008). Even though acute stress did not affect gut histology of Atlantic cod, there was an increase in the intestinal permeability and partial alterations in intestinal microbiota.

More recently, other authors evaluated the effects of chronic stress on the intestinal mucosal immune system (Niklasson et al., 2011) and Intestinal barrier function (Sundh et al., 2009; Sundh et al., 2010) in Atlantic salmon. These studies demonstrated that as well as acute stress, chronic stress also involves significant alterations in the intestine of salmonids. Furthermore, Sundh *et al.* (2010) suggested the use of the intestinal barrier function as a marker to assess chronic stress in Atlantic salmon, after reporting that is possible to detect different morphological and functional changes in the intestine of fish subjected to long-term stressful conditions.

Even though there is evidence of the influence of acute and chronic stress on the function of GI tract in fish, there is still scarce information about the effect of stress on the intestinal microbiota. It is not clear which mechanisms modulate the bacterial population level in fish submitted to stress conditions. However, some authors suggest that a peel-off effect of intestinal mucus may lead to the decrease in the number of adherent microbes in the gut and subsequent increase in the faeces after stress conditions, (Olsen et al., 2002; Olsen et al., 2005).

This last topic deserves further research since the mentioned studies only made use of conventional microbiological techniques based on culture methods to detect intestinal microbiota. This approach does not reflect the non-culturable and obligate

anaerobic microbiota and thus underestimates the potential impact on the entire community. Several studies have noted the importance of using culture - independent techniques such as molecular methods to evaluate microbiota, revealing that the use of culture methods only provides no information on non-culturable organisms that could also play a major role in the intestine (Pond et al., 2006; Zhou et al., 2014).

1.3.3. Can probiotics mitigate the stress effects on fish?

The beneficial effects of probiotic administration are well documented in fish. Several authors have reported significant evidence of beneficial microbes with potential to improve growth performance, immune system, disease resistance, and health status (reviewed by (Lauzon et al., 2014; Pérez-Sánchez et al., 2014; Akhter et al., 2015)). Thus, probiotic supplementation of diets, which potentially may improve farmed fish conditions, has gained significant attention (Tinh et al., 2008; Dimitroglou et al., 2011; Lazado and Caipang, 2014a). Besides these previously known effects of probiotic in fish, recent studies have reported that different probiotics species may ameliorate the adverse effect of stress in fish (Table 1.1.). However, according to Mohapatra et al. (2013), there have been only a few studies that have tried to find the mechanism in which probiotic modulates stress in fish.

Table 1.1. Studies assessing the effect of probiotic on fish stress responses.

Probiotic	Fish species	Effect of probiotic on stress parameter evaluated	References
L. rhamnosus	*Amphiprion ocellaris*	Lower gene expression of glucocorticoid receptor and *hsp70*	(Avella et al., 2010b)
B. subtilis, B. licheniformis and B. pumilus	*Sparus aurata*	Lower gene expression of glucocorticoid receptor and *hsp70*	(Avella et al., 2010a)
E. faecium	*Solea solea*	Lower gene expression of *hsp70* Increased cortisol levels	(Avella et al., 2011)

L. delbrueckii delbrueckii,	*Dicentrarchus labrax,* L.	Decreased cortisol levels	(Carnevali et al., 2006)
B. subtilis, B. licheniformes, L. acidophilus and S. cerevisiae	*Carnegiella strigata*	Decreased cortisol levels	(Gomes et al., 2008)
B. subtilis, B licheniformes, L. acidophilus and S. cerevisiae	*Paracheirodon axelrodi*	Decreased cortisol levels Increased survival after transportation	(Gomes et al., 2009)
B. subtilis, Lactococcus lacti and S. cerevisiae	*Labeo rohita*	Increased of antioxidant enzymes Low glucose levels Less histopathological changes in gills and liver after fenvalerate exposure	(Mohapatra et al., 2012)
E. faecium	*Solea*	Increased cortisol levels Modulation of gene expression of hypothalamic–pituitary-interrenal axis	(Palermo et al., 2011)
L. fructivorans and L. plantarum	*Sparus aurata*	Decreased cortisol levels and mortality after pH stress test High gene expression of *hsp70*	(Rollo et al., 2006)
B. subtilis, L. acidophilus, C. butyricum and S. cerevisiae	*Paralichthys olivaceus*	High survival after pathogen challenge tests. Increased of tolerance after stress tests	(Taoka et al., 2006)
S. cerevisiae	*Ictalurus punctatus*	Low cortisol and lactate levels after low-water stress	(Welker et al., 2007)

L.= Lactobacillus, B. = Bacillus, E. = Enterococcus, S = Saccharomyces, C = Clostridium, hsp70 = 70 kDa Heat Shock Protein gene expression

Probiotics have been demonstrated to be effective in decreasing the adverse effects of stress caused by transport (Gomes et al., 2008; Gomes et al., 2009), pesticide (fenvalerate) (Mohapatra et al., 2012), adverse pH (Rollo et al., 2006), heat shock and pathogen challenge (Taoka et al., 2006) and low water level (Welker et al., 2007). The ability of probiotics to ameliorate the negative effect of stress could become a valuable tool to improve the welfare of fish under culture conditions. Studies in mammals using GF and specific pathogen-free mice as a model suggest that the gut microbiota could modulate the hypothalamic-pituitary-adrenal response to stress (Sudo, 2006).

Some authors have reported that probiotics can modulate stress markers such as cortisol levels and gene expression of *hsp70*, glucocorticoid receptor and genes related with the hypothalamic-pituitary-interrenal axis (Rollo et al., 2006; Avella et al., 2010a; Palermo et al., 2011). Nonetheless, the correlations of stress markers are not always consistent. For instance, a study carried by (Rollo et al., 2006) reported that *S. aurata* fed a diet supplemented with a probiotic had a decrease of cortisol level and higher gene expression of *hsp70* after a pH stress test in comparison with the control group fed the same diet without probiotic. Controversially, these results differ from the study reported by Avella et al. (2011), who demonstrated that the use of *E. faecium* as probiotic in *S. solea* resulted in an increase of cortisol levels accompanied with low gene expression of *hsp70*. Therefore, further research is necessary to understand how stress markers are regulated by probiotics.

Since only a few of the previously mentioned studies have investigated whether probiotics can modulate the gut microbiota and the intestinal barrier function, a holistic approach using different techniques such as histology, electronic microscopy and metagenomic techniques is recommended in order to gain a wider understanding of the relations between stress, probiotics, gut microbiota and the intestinal barrier.

1.3.4. Use of Pediococcus acidilactici in salmonids

Pediococcus acidilactici is a Gram-positive cocci that belongs to the lactic acid bacteria (LAB) group, and is closely related to *Lactobacillus casei/paracasei* as described by (Holzapfel et al., 2006). Members of the *Pediococcus* genus are homofermentative and use glucose to produce lactic acid but not CO_2. The typical cell morphology of species belonging to the *Pediococcus* genus differs from all other LAB due to the spherical shape (0.5-0.8 µm) and the ability to divide into two planes at right angles to

form tetrads. In contrast with other LAB such as *Leuconostoc* and *Streptococcus*, *Pediococcus* never form chains. However, *Pediococcus* can also be found in pairs or single cells. The growth of *P. acidilactici* occurs in a wide range of conditions and is considered a facultative aerobic able to growth under microaerophilic conditions (Holzapfel et al., 2006).

P. acidilactici have been important in the food industry due to its positive role in the fermentation of different foods and alcoholic beverages where often is associated with other LAB. Several authors have reported the presence of *P. acidilactici* in the GI tract of various animals including birds, fish and freshwater prawns (Holzapfel et al., 2006).

In aquaculture, *P. acidilactici* has been used as a probiotic under the product name Bactocell®, which contains the single live strain MA18/5M. This strain was isolated in France from natural pasture-Gramineae (Barreau et al., 2012). The use of Bactocell® in animal feed as a probiotic is well documented in different animal species including pigs and chicken (Di Giancamillo et al., 2008). The use of Bactocell® in aquaculture as a probiotic for salmonids and shrimps was approved in 2009 by EFSA based on studies reporting its beneficial effects to improve vertebral malformations in rainbow trout as well as improvement of growth and disease resistance in shrimps. Subsequently, EFSA approved Bactocell® use for other fish species, and currently, this product is the only live microorganism approved in the European Union to be used as a probiotic in fish. The first study using *P. acidilactici* in fish was probably conducted by Gatesoupe (2002) in *Pollachius pollachius.* Several subsequent studies have reported the beneficial use of this microorganism in different aquatic species (reviewed by Merrifield and Carnevali (2014)).

The use of *P. acidilactici* in salmonids (rainbow trout and Atlantic salmon) is well documented. Nonetheless, the number of studies in rainbow trout outnumbers the research carried out in Atlantic salmon as only two studies have been reported so far. The studies evaluating the effect of *P. acidilactici* in Atlantic salmon and rainbow trout are summarised in Table 1.2. These studies suggest that *P. acidilactici* can positively influence the health of salmonids under different conditions. However, the mechanisms by which this bacterium induce the beneficial effects on fish have not to date been fully demonstrated.

Table 1.2. Studies using *Pediococcus acidilactici* in salmonids.

Species	Stage/ duration of administration	Route of administration and dose	Parameters investigated	Main effects observed	Reference
Atlantic salmon (*Salmo salar*)	250 ± 13 g / 63 days	Diet[1] 0.035% (3.03 x 10^6 cfu g^{-1}).	GM, GH, IR, GP, gene expression antiviral response	↓total bacterial count ↑ microbial diversity digesta anterior ↑ Villi length ↑ IELs ↑ IL1b, TNFa, IL8, TLR3 and Mx-1 → Growth performance ↑Serum lysozyme activity	(Abid et al., 2013)
	150 g / 21 days	Dietary (1.0 x 10^7 cfu g^{-1})	GH Gene and protein expression inflammatory response in distal intestine	↑ Goblet cells, intraepithelial lymphocytes, supranuclear vacuoles and immune cell in lamina propria ↓ expression of genes related to inflammatory response ↑ recovery of inflammatory challenge Modulation of expression of proteins in distal intestine	(Vasanth et al., 2015)
Rainbow trout (*Oncorhynchus mykiss*)	Larval stage / 20 days group B1 and 5 months group B5	Diet (1.5 ± 0.4 x10^{-6})	SR, VM, GP, GM	→ Growth performance → Survival ↓malformed fish fed group B5 → Aerobic bacterial counts hindgut	(Aubin et al., 2005)
	240-250 g / 14 days	Diet[2] (1.0 x10^{-7})	PA with *Vibrio anguillarum,* GM, GH	↑ leukocytes levels and goblet cells. ↓ Reduction of	(Harper et al., 2011)

			epithelial tissue damage *P. acidilactici* colonised and outcompeted *V. anguillarum* in the intestinal mucosa	
15.04 ± 0.52 g / 8 weeks	Diet[1,2] (3.71 x10^{-7})	AEA, DR to Staphylococcus iniae	↑ Catalase, glutathione S-transferase, glutathione reductase ↑ Resistance to *S. iniae* challenge	(Hoseinifar et al., 2016)
15.04 ± 0.52 g / 8 weeks	Diet[1,2] (3.71 x10^{-7})	GP, GM, HP	→ GP probiotic group ↑ GP synbiotic group → HP ↑ Total aerobic bacteria and presumptive autochtonous LAB	(Hoseinifar et al., 2015a)
15.04 ± 0.52 g / 8 weeks	Diet[1,2] (3.71 x10^{-7})	IR, PA, DR	↑ Serum alternative complement activity ↑ Respiratory burst activity ↑ Skin mucus protein ↑ Bactericidal activity	(Hoseinifar et al., 2015b)
Day 1 larval stage / 7 weeks	Diet (7 × 10^{-5} cfu g^{-1})	GM, IR	No modulation of gut microbiota Minor up-regulation in transcription of immune genes (MBL 2, CD8 and FOXP3b).	(Ingerslev et al., 2014b)
9 g / 10 weeks	Diet (2.88 × 10^{7} cfu g^{-1} and 1.28 × 10^{8} cfu g^{-1})	GP, GM, BC, IR, FU	↑ *K*-factor ↑ leukocytes count Colonization of intestinal mucosa	(Merrifield et al., 2011)

	100 g / 5 weeks	Diet (1×10^7 cfu g^{-1})	GH, GM	↑ Microvilli length in the proximal intestine ↑ Endocytic activity in the proximal and distal intestinal mucosa	(Merrifield et al., 2010d)
	16.4 ± 0.4 g / 8 weeks	Diet (low doses 2.6 x 10^4 cfu g^{-1}; high doses 7.2 x 10^4 cfu g^{-1})	BC, GP, IR, GI, GH, FU	→-BC ↑ feed conversion and protein efficiency in high doses group ↑ Dry matter and protein retention in high doses group ↑ Alternative complement activity	(Ramos et al., 2015)
	16.4 ± 0.4 g / 56 days (GP) / 96 days (GM)	Dietary (low doses 2.6 x 10^4 cfu g^{-1}; high doses 7.2 x 10^4 cfu g^{-1})	GP, GM	→ GP ↑ Diversity (Shannon index) in low doses group	(Ramos et al., 2013)

GH - gut histology (inclusive electronic microscopy), GM - gut microbiota, GP - growth performance, SR - survival rate, PA - pathogen antagonism, VM - vertebral malformation, IR - immunological response, HP - haematological parameters, DR - disease resistance, AEA - antioxidant enzymes activity, BC - body composition, FU - feed utilization

[1] Used in a synbiotic

[2] *Ex-vivo*

1.4. Thesis aims and objectives

The aim of this research programme was to evaluate the effect on the intestinal microbiota of challenging conditions that salmonids encounter during the farming process and to evaluate the potential beneficial influence of the probiotic *Pediococcus acidilactici* MA18/5M.

In order to achieve the main aim of this project four objectives were formulated:

Objective 1: Improve the knowledge of the bacterial microbiota in the intestine of salmonids.

Objective 2: Determine the effect that different stressors have on the intestine and associated microbiota.

Objective 3: Investigate if *P. acidilactici* can promote beneficial effects on the intestine of salmonids and mitigate the adverse effects of challenging farming events.

Objective 4: Evaluate the interaction between *P. acidilactici* and bacterial microbiota in the intestine.

CHAPTER 2. GENERAL MATERIALS AND METHODS

2.1. Overview

This chapter describes the general procedures and analytical techniques used in the different experiments presented in this thesis. Experimental design, diet formulation and other methods unique to specific experiments are described in detail in each chapter and therefore are not presented here. The experiments conducted in this research programme were carried out on two salmonid species; rainbow trout (*Oncorhynchus mykiss*) and Atlantic salmon (*Salmo salar*). A summary of the experimental conditions for each experiment is presented in Table 2.1. All experimental work was conducted in accordance with the Plymouth University ethics committee and approved by the different national authorities according to the place where the experiment was carried out.

Table 2.1. General conditions of experiments.

Experiment/Chapter	Fish	Duration experiment	Aquarium system	Location
1/Chapter 3	Rainbow trout	12 weeks	Freshwater recirculation system	Plymouth, UK
2/Chapter 4	Rainbow trout	4 weeks	Freshwater recirculation system	Plymouth, UK
3/Chapter 5	Atlantic Salmon	10 weeks	Freshwater and seawater open system	Tromsø, Norway
4/Chapter 6	Atlantic Salmon	12 weeks	Seawater recirculation system	Hirtshals, Denmark

2.2. Measurement of growth related parameters

Growth performance and feed utilisation were assessed using the following parameters; percentage wet weight gain (WG), specific growth rate (SGR) and feed conversion ratio (FCR). Parameters were calculated using the following formulae:

WG (g/ fish) = FW-IW

SGR (%) = (Ln FW-Ln IW) / t x 100

FCR = FI / WG

Where FW is the final weight (g), IW is the initial weight (g), t is the number of feeding days, FI is feed intake (g), WG is wet weight gain, Σ Temp is the sum of average daily temperatures (°C) and Ln = natural logarithm value.

2.3. Fish dissection, sampling and samples storage

Fish were euthanised by immersion in overdose (200 mg L^{-1} water for 15 min) of buffered tricaine methane sulfonate (MS222; Pharmaq, Fordingbridge, UK) followed by the destruction of the brain. Only fish with digesta content throughout the intestine were sampled to ensure exposure to the diet. Under aseptic conditions, fish was opened by the mid-line, and the entire intestinal tract was dissected and adipose tissue removed. The intestine was divided into proximal, mid and distal intestine as described in Figure 2.1. However, not all regions were sampled in each experiment. In order to be consistent during the samplings, each set of samples for a specific analysis were always taken from the same intestinal region of all fish. For histological analysis, an intestinal portion of approximately 0.5 mm was excised and placed into a tube with 10% buffered formalin for 48 hours and then transferred to 70% ethanol. For microbiological analysis, digesta was obtained from mid or distal intestinal region separately by gentle squeezing the intestine with a sterile forceps into individual sterile 1.5 ml microcentrifuge tubes. Mucosal tissue was washed thoroughly three times with sterile phosphate-buffered saline (PBS; pH 7.3), and a portion of 0.5 mm of the intestine was excised and kept in sterile 1.5 ml tubes. For proximal intestine, two pyloric caeca were excised from the base, and the whole structure (mucosa and digesta) was stored without washing with PBS. Samples for microbiological analysis were snap-frozen in

liquid nitrogen, transported on dry ice and subsequently stored at -20 °C until DNA extraction. For gene expression analysis mucosa samples were taken of each region; 0.5 mm from the mid and distal intestine and two pyloric caeca from the proximal intestine. Thereafter, samples were immersed in RNALater (Ambion, Carlsbad CA, USA) at a ratio of 1:4, transported at room temperature for 24-48 h and then stored at -80 °C until RNA extraction.

Figure 2.1. Intestinal regions used for sampling.

2.4. Microbiota analysis

2.4.1. DNA extraction and polymerase chain reaction

DNA was extracted from digesta or mucosa using the QIAamp® Stool Mini Kit (Qiagen, Crawley, UK) following the modified protocol summarised by (Falcinelli et al., 2015) which involves cell lysis, inhibitor removal, protein removal, precipitation, cleaning and DNA recovery. All steps were performed with sterile and molecular grade reagents. Centrifugation was always done at maximum speed (17,000 x *g*). Lysozyme solution (500 µl of 50 mg ml^{-1} in TE buffer) was added to each sample and then incubated for 30 min at 37°C to enhance lysis of Gram-positive bacteria. Subsequently, 800µL of ASL buffer was added, and then the sample was vortexed and incubated for 10 min

at 90°C followed by centrifugation for 1 min. To remove inhibitors, the supernatant was transferred to a new tube containing a half tablet of inhibitEX provided by the kit, thereafter the sample mixture was vortexed, incubated for 1 min at room temperature and then centrifuged for 3-4 min. A volume of 230 µl of the supernatant was pipetted into a new tube and subsequently 230 µl of AL buffer and 20 µl of proteinase K were added to the tubes, which were incubated at 56°C for 60 mins. After incubation, 460 µl of ice-cold phenol solution was added to the sample, then mixed by hand and incubated on ice for 10 min. Thereafter, 460 µl of chloroform was added to the tubes and the sample centrifuged for 5 min. The upper aqueous layer was carefully pipetted into a new tube, and the chloroform step repeated. To precipitate the DNA, 230 µl of ice-cold isopropanol and 96 µl of sodium acetate 3 M were added and the sample incubated at -20°C overnight. DNA recovery was done by centrifuging the sample for 15 min at 4°C. The DNA pellet was washed with 70% ethanol twice and air dried for 10 mins and then diluted in 30 µl of buffer TE. The DNA quality and yield was checked spectrophotometrically (Nanodrop 2000 Thermo Scientific, Wilmington, USA).

Polymerase chain reaction (PCR) was performed in duplicate targeting the hypervariable region V1-V2 of the bacterial 16S rRNA gene using primers reported by Roeselers et al. (2011) as follows: forward primer 27F (5'-aga gttt gat cmt ggc tca g-3'), reverse primers 338R-I (5'-gcw gcc tcc cgt agg agt-3') and 338R-II (5'-gcw gcc acc cgt agg tgt-3'). Reverse primers were mixed and used at the same equimolar concentrations as that of forward primer. Primers were synthesised by Eurofins MWG (Ebersberg, Germany). All PCR reactions were performed using GeneAmp® PCR System 9700 (Perkin-Elmer, CA, USA). PCR reactions were carried out using 25 µl BioMix™ Red Taq (Bioline, UK), 0.5 µl of each primer (50 pmol/µl), 1 µl DNA template and adjusted to a final volume of 50 µl molecular biology-grade water. Each reaction

included a negative control (sterile, molecular grade water as template). A touchdown PCR was conducted at the following conditions: initial denaturation at 94°C for 7 min, then 10 cycles of 94°C for 30 s, 63°C for 30 s (decreasing 1°C every cycle) and 72°C for 30 s; this was followed by 25 cycles of 94°C for 30 s, 53°C for 30 s and 72°C for 30 s; final extension at 72°C for 10 min. The PCR products were checked for size and specificity by electrophoresis on 1.5 % w/v agarose gel. The duplicate PCR reactions were combined and purified with QIAquick PCR Purification Kit (Qiagen, Valencia, CA), following manufacturer's protocol. Purified samples were evaluated with Bioanalyzer previous to amplicon library preparation.

2.4.2. Amplicon library and sequencing

Prior to Ion Torrent PGM sequencing, the amplicons were assessed for fragment concentration using an Ion Library Quantitation Kit (Life Technologies™, USA), then concentrations were adjusted to 26 pM. Amplicons were attached to Ion Sphere Particles using Ion PGM Template OT2 400 kit (Life Technologies™, USA). Sequencing was performed with Ion Xpress Barcode Adapters (1-16 Kit; Life Technologies™) and a 318™ chip (Life Technologies™) on an Ion Torrent Personal Genome Machine (Life Technologies™). Sequences were binned by sample and quality filtered within the PGM software (Torrent Suite™ software life Technology) to remove polyclonal and low-quality reads. Fastq files for each sample were exported for the subsequent bioinformatics analysis.

2.4.3. High-throughput sequence analysis

The quality and number of reads for each sample were assessed using FASTQC v0.11.4 (http://www.bioinformatics.bbsrc.ac.uk/projects/fastqc/). Raw sequences were

filtered by quality using FASTXToolkit (http://hannonlab.cshl.edu/fastx_toolkit). Only sequences with at least 80% of the sequence had a minimum acceptable Phred quality score of >20 were retained. Reads which passed all quality control steps were concatenated into a single FASTA file for subsequent processing. Filtered quality sequences were analysed using the Quantitative Insights Into Microbial Ecology (QIIME) software version 1.8.0 (Caporaso et al., 2010b). Sequences were clustered in OTUs using a 97% sequence similarity threshold using open-reference OTU picking approach with USEARCH pipeline version 6.1 (Edgar, 2010). This pipeline involves clustering, chimera checking, and quality filtering. The taxonomy was assigned using RDP classifier (Wang et al., 2007) and Greengenes database gg_13_8_otus (DeSantis et al., 2006). The OTUs representative sequences were aligned using Pynast (Caporaso et al., 2010a) with a minimum sequence length threshold of 150 bp. A phylogenetic tree was constructed with FastTree (Price et al., 2010) Finally, the resulting OTU table was filtered at 0.005% to remove singletons (OTUs represented by only a single sequence) and reduce spurious OTUs (Navas-Molina et al., 2013; Flynn et al., 2015). In addition, the reads classified as Streptophyta was removed from the dataset and not included in the analyses as member assigned to this group are considered to be contamination from diet and water and not part of the gut microbiota (Zarkasi et al., 2014; Estruch et al., 2015). The core microbiota was calculated in QIIME and defined as the OTUs shared in 80% of each experimental group. A Venn diagram representing the core microbiota was constructed in http://bioinfogp.cnb.csic.es/tools/venny/index.html (Oliveros, 2007). Diversity metrics analyses were performed in QIIME rarefying all the samples at a depth of the least number of sequences throughout the samples. Alpha diversity of each sample was calculated using three metrics: Chao1, Observed species and whole-tree phylogenetic

diversity. Beta diversity was determined between samples with weighted and unweighted UniFrac (Lozupone and Knight, 2005). PCoA plots from beta diversity results were visualised with EMPeror (Vázquez-Baeza et al., 2013).

2.5. Intestinal gene expression

Table 2.2. summarise the set of genes used for gene expression in Atlantic salmon, in Chapter 5 and Chapter 6 to evaluate the effect of *P. acidilactici* and the replacement of fish oil by vegetable oil in the intestinal health.

Table 2.2. Genes used for gene expression analysis.

Gene – gene symbol	Encoding protein	Specific functions	Studies in salmonids
il-1b	interleukin-1b	Pro-inflammatory cytokine with effect in inflammation and immune defense response	(Lilleeng et al., 2009)
anx a1	Annexin-a1	Anti-inflammatory properties by inhibiting biosynthesis of eicosanoids.	(Vasanth et al., 2015)
il-17a	Interleukin-17a	IL-17A is a pro-inflammatory cytokine produced predominantly by activated T cells.	(Marjara et al., 2012)
tnf-a	Tumor necrosis factor-a	Pro-inflammatory cytokine with effect in inflammation and immune defense response.	(Vasanth et al., 2015)
ifn-a	Interferon-a	Inhibit virus replication and modulate immune response.	(Niklasson et al., 2014)
mx1	Mx1 protein	Antiviral activity against a wide range of RNA viruses and some DNA viruses.	(Niklasson et al., 2014)
tlr-3	Toll-like receptor-3	Activation of antiviral immune response through recognition of double-stranded RNA	(Abid et al., 2013)
hsp-70	70-kDa heat shock protein	Regulation of stress tolerance and induce anti-apoptotic activity under cellular stress conditions	(Bakke-McKellep et al., 2007)
pcna	Proliferating cell nuclear antigen	Regulation of cell cycle as well as participation in the synthesis and reparation of DNA.	(Gajardo et al., 2016a)
aqp-8ab	Aquaporin-8ab	Intestinal water absorption	(Engelund et al., 2013)
claudin-15 *claudin-25b* *occludin* *jam-1b*	Claudin-15 Claudin-25b Occludin Junctional adhesion molecule-1B	Formation and regulation of the tight junction	(Hu et al., 2016)
e-cadherin	Epithelial cadherin	Mechanical integrity of intestinal epithelium	(Hu et al., 2016)

2.5.1. RNA extraction and cDNA synthesis

RNA from intestine sections was extracted using TRI reagent (Sigma-Aldrich, Poole, UK) according to the manufacturer's instructions, with some modifications. Briefly, 30-50 mg of tissue were removed from RNAlater, immersed into TRI reagent, homogenised by vortex for 30 s and incubated for 10 min at room temperature. Then, 200 µl of chloroform were added to the tubes, shaken vigorously for 15 seconds and incubated at room temperature for 10 mins. The samples were centrifuged at 12,000 x *g* for 15 min. The upper aqueous phase was transferred into a tube containing an equal volume of molecular grade isopropanol. The mixture was then vortexed and centrifuged at 12,000 x *g* for 15 min at 4-8°C. The supernatant was discarded, and the precipitated RNA pellets were washed using 1 ml of 75% ethanol. The samples were gently mixed by hand and then centrifuged at 10,000 x *g* for 10 min at room temperature. After the final wash, the ethanol was removed, and the pellets were air-dried for 5 – 10 min and then resuspended in 30 µl of diethylpyrocarbonate (DEPC) water. To remove any contaminating genomic DNA, the RNA was treated with DNase (TURBO DNA-free™, Ambion) following the manufacturer's instructions. The yield and quality of RNA in each sample were determined by measuring 260/280 nm and 260/230 absorbance ratios (NanoDrop Technologies, Wilmington, USA). The integrity of RNA was confirmed by running the RNA extracted from the samples in a 1 % agarose gel. RNA samples were stored at -80 °C. A total amount of 1 µg of RNA was used for cDNA synthesis, using iScript™ cDNA synthesis kit (Biorad, CA, USA) following manufacturer's instructions. For each set of samples, a negative control was included by performing a reaction with a pool of randomly selected RNA from samples of each experiment without the reverse transcriptase enzyme to control genomic DNA

contamination. The synthesised cDNA and negative controls were diluted in molecular grade water and stored at -20°C.

2.5.2. Quantitative real-time polymerase chain reaction (qPCR)

All Quantitative real-time PCR (qPCR) reactions were performed with the SYBR green method using a StepOne Plus™ Real-time PCR thermal cycler (Applied Biosystems, Life Technologies) and with the QuantStudio® 12K Flex Real-Time PCR system (Applied Biosystems, Life Technologies). Duplicate qPCR reactions were set on 384-well or 96-well plates by mixing 2.0 µl of cDNA template (1/10 or 1/20 dilution according to the experiment), 3.75 µl iTaq™ Universal SYBR® Green Supermix (Bio-Rad, CA, USA), 0.225 µl of forward and reverse primer (0.3 µM) and 1.3 µl of molecular grade water (Ambion). The thermal profile for all reactions was 10 min at 95 ºC and then 40 cycles of 15 s at 95 ºC, 60 s at 60ºC. Fluorescence monitoring occurred at the end of each cycle, and additional melting curve analysis was performed using a temperature range of 60 °C to 95 °C at 0.3 °C intervals. For each set of samples and genes evaluated two controls were used. First, a no-template control to ensure the absence of DNA contamination in the reagents and environment and second, a no reverse transcription control prepared during cDNA synthesis as previously described.

2.5.3. Primer Optimization

All the primers for gene expression were synthesised by Eurofins MWG (Ebersberg, Germany). Primer sequences were designed using Primer3 (Rozen and Skaletsky, 1999) or obtained from previous publications. Primer specificity for reference and target genes were evaluated *in silico* by the tool Primer-BLAST (Ye et al., 2012) available at http://www.ncbi.nlm.nih.gov/tools/primer-blast/. Specificity was also

checked by a melting curve after each qPCR assay and subsequent agarose gel electrophoresis to confirm the amplification of a single product with the expected molecular size and absence of primer-dimers. Amplification efficiency (*E*) was determined for each primer set using a standard curve based on five dilution series from cDNA (1:4 or 1:10), which was prepared by pooling an equal amount of cDNA from a representative number of samples from the same intestinal region. Each dilution was run in triplicate, and linear regression of the standard curve was constructed with quantification cycle (C_q) values; R-squared (R^2) and slope were also calculated. The amplification efficiency was calculated with the formula: ($E = 10^{(1/-\text{slope})} - 1$). R^2 values and *E* for all primer sets were >0.97 and 1.83-2.04, respectively. For information relating to the primer sequences, qPCR efficiency, primer annealing, amplicon sizes, references and target genes, refer to individual chapters.

2.5.4. Data analysis

The raw C_q values for reference and target genes were exported to Microsoft Excel and corrected by qPCR efficiency. Reference genes were chosen by ranking them according to overall coefficient variation and their interspecific variance as described by (Kortner et al., 2011). Gene expression for each gene was normalised to the geometric average expression of at least two stable reference genes using corrected raw C_q. Normalised gene expression of each target gene was calculated from corrected raw C_q (Pfaffl, 2001).

2.6. Statistical analysis

To investigate experimental group differences between bacterial communities (beta diversity), the software package PRIMER-E v.6 was used (PRIMER-E, Plymouth,

UK)(Clarke and Gorley, 2006). Beta diversity was calculated using UniFrac. Weighted and unweighted UniFrac were calculated in QIIME, and dissimilarity matrixes were imported to PRIMER-E to evaluate significant differences between groups by permutational multivariate analysis of variance (PERMANOVA). Differences in relative abundance of OTUs between groups were analysed with LEfSe (Segata et al., 2011), available at http://huttenhower.sph.harvard.edu/galaxy/ using the default parameters. This tool first identifies significant differences among experimental groups and then evaluates whether these differences are consistent with other features; for example, the phylogenetic affiliation of the OTUs. LEfSe implements different statistics test involving firstly, a non-parametric factorial Kruskal-Wallis rank sum test; secondly, a pair-wise test using Wilcoxon sum-rank test; and finally, linear discriminant analysis (LDA) to estimate the effect size of each differentially abundant OTU. The results of all other analysis presented in this thesis (i.e. growth related parameters, gene expression, histological evaluation and alpha diversity) were analysed using SPSS version 18 (SPSS Inc., Chicago, IL, USA). The data were checked for normality (Shapiro-Wilk test) and homogeneity of variance (Levene). Data fulfilling parametric test assumptions were analysed either by t-test or analysis of variance (ANOVA). Pair-wise comparison was performed to detect differences between individual treatments using Tukey-Kramer HSD post hoc test. Data that did not fulfil parametric test assumptions were log transformed to achieve normality or otherwise analysed with a non-parametric test such as Mann-Whitney *U* test or Kruskal-Wallis. All data are presented as mean ± standard deviation (SD), and significance was accepted at $P < 0.05$.

CHAPTER 3. Evaluation of *Pediococcus acidilactici* in rainbow trout under different inclusion levels of alternative plant proteins sources in the diet: a microbiota study

3.1. Abstract

This study aimed to assess the effect of the probiotic *P. acidilactici* MA18/5M and different plant ingredients on the intestinal microbiota of rainbow trout and to evaluate whether the effect of the probiotic supplementation is influenced by the various feed ingredients. A 12-week feeding trial was conducted in a freshwater recirculation system. The experimental designed consisted of six experimental groups i.e. three dietary groups with two treatments each, i.e. control and probiotic Bactocell® (at 10^6 CFU/g *P. acidilactici* MA18/5M). The basal diets were as follows: the fishmeal diet (diet FM), the soybean diet (diet SB) and the plant mix diet (diet PMIX) containing a mix of vegetable proteins. Samples were taken from the distal intestinal digesta to characterise the microbiota by high-throughput sequencing, growth performance was also evaluated. No significant differences were evident in growth performance between treatments (control and probiotic groups) in any of the basal diets. The microbiota analysis showed differences in both alpha and beta diversity between the control diets and the diets with probiotic supplementation and these changes were diet-dependent. Beta diversity analysis showed that the basal diets were the primary factor influencing the modulation of the gut microbiota. A core of shared microbiota was composed by 66 OTUs, which represented >60% of the total relative abundance in all the experimental groups. This study showed that specific ingredients in the diet formulation not only modulate the intestinal microbiota of rainbow trout but also can affect the viability of *P. acidilactici* during feed production. The result of this study also suggest that different diet ingredients differ in the extent of the effect of *P. acidilactici* on the intestinal microbiota.

3.2. Introduction

The salmonid aquaculture industry has expanded considerably in the last year 20 years, which has brought important challenges for the feed industry. Traditionally, aquafeed for salmonid species has relied on marine ingredients from wild-caught fish to provide their high protein requirements (Tacon and Metian, 2008; Ytrestøyl et al., 2015). However, wild capture fisheries have remained relatively static since 1990 (FAO, 2014). A limited availability of wild-caught fish as a source of fishmeal for salmonids diets, together with the expansion of the salmonid aquaculture production, have encouraged the salmonid industry to search for more sustainable sources of proteins. Thus, plant-based ingredients are increasingly being used as major feed ingredients in aquafeeds to replace fishmeal.

Currently, different plant feedstuffs have been incorporated into the diets of commercially important salmonid species such as rainbow trout (reviewed by (Ytrestøyl et al., 2015)). However, the use of plant feedstuff in salmonids fish diets is restricted mainly by the presence of antinutritional factors in plants (Krogdahl et al., 2010). In particular, high dietary inclusions of soybean meal (SBM) have been reported to induce damage at different levels of the intestinal mucosa in salmonids, leading to enteritis and other pathologies (De Santis et al., 2015; Krogdahl et al., 2015)). SBM and other plant protein ingredients are also able to affect the intestinal microbiota in salmonids, but a link between the modulation of the intestinal microbiota and the development or causality of enteritis remains unclear (Bakke-McKellep et al., 2007).

Probiotics are live microbial organisms which, when supplied in the environment or into the feed, confer benefits to the host. Potential benefits of probiotic microorganisms provided in the diet depend on a broad range of conditions. The viability and

metabolism are important factors that influence the ability of probiotics to produce the beneficial effect in the host. The mentioned factors are dependent on substrate available in the GI tract. Different diet ingredients could modulate directly the activity of microorganisms in the intestine including the probiotic microorganisms, or indirectly affecting the interaction of such microorganism with the intestinal barrier. The lactic acid bacterium *P. acidilactici* MA18/5M has been demonstrated to have a number of beneficial effects in rainbow trout leading to improvements in the gut health (Aubin et al., 2005; Merrifield et al., 2010d; Ramos et al., 2013; Ramos et al., 2015). However, the interaction between this probiotic bacterium and different feed ingredients in the diets has not been comprehensively studied in fish. Thus, the aims of this study were 1) to assess the effects of *P. acidilactici* MA18/5M and different plant ingredients on the intestinal microbiota of rainbow trout and 2) to evaluate whether the probiotic supplementation is affected by different feed ingredients.

3.3. Materials and Methods

3.3.1 Animal husbandry

The 12-week-feeding trial was conducted at the recirculation aquarium facilities at Plymouth University, UK in accordance with the university ethical committee and under the UK Home Office project licence PPL 30/2644. Prior to initiation of the trial, juvenile rainbow trout (*Oncorhynchus mykiss* Walbaum) were acclimated for three weeks on a standard commercial diet (Sigma® 50, EWOS, UK). At the end of the acclimatisation period, 360 fish (48.9 ± 0.3 g) were randomly distributed within eighteen fibreglass tanks (80 L capacity) in a density n = 20 trout per tank. The fish were kept in a freshwater recirculation system (flow rate 100 L/h) with a 12-h dark: 12-h light photoperiod. During the experimental period, the dissolved oxygen level was maintained at 81.2 ± 3.9 % saturation; the temperature was 15.1 ± 0.9 °C and the pH 6.8 ± 0.6. Additionally, ammonia, nitrite and nitrate were measured weekly and maintained at 0.1 ± 0.02 mg L^{-1}, 0.04 ± 0.01 mg L^{-1} and 15.6 ± 5.9 mg L^{-1}, respectively by gradual water changes. Throughout the experiment, feed input was supplied in equal rations three times per day (09:00, 13:00 and 18:00), seven days a week. The feeding rate was adjusted daily based on a predicted growth, assuming a FCR of 1, and varied from 1.5% to 2.2% body weight. Fish were weighed (in bulk, by tank) at the start of the trial and afterwards every two weeks until week twelve.

3.3.2. Feed formulation and experimental design

Three different “basal” diets were formulated to be iso-lipidic (20%) and iso-nitrogenous (50%). The fishmeal diet (FM) contained fishmeal as the main protein source; the plant diets were formulated to replace 62.7% of fishmeal by plant-based

protein ingredients. The main ingredients used for replacing fishmeal in the plant diets were SBM and soybean protein concentrate for the soybean diet (SB) and soybean meal, soybean protein concentrate, pea protein, corn gluten meal and gluten wheat for the plant mix diet (PMIX). The feed ingredients and proximate composition of the experimental diets are shown in Table 3.1. Two batches of each diet were manufactured, one served as the control, and the another one was supplemented with the probiotic Bactocell® (at 10^6 CFU/g *P. acidilactici* MA18/5M). This resulted in six experimental groups i.e. three dietary groups with two treatments each i.e. control and probiotic. Each experimental group had three replicate tanks.

Table 3.1. Formulation of experimental diets and chemical composition.

	Fishmeal (FM)		Soybean meal (SBM)		Plant Mix (PMix)	
Ingredients (%)	**Control**	**Probiotic**	**Control**	**Probiotic**	**Control**	**Probiotic**
Fishmeal LT94ϕ	670.7	670.7	250	250	250	250
Soya HP 48¥	-	-	398.4	398.4	189.7	189.7
Soya SPC 60¥	-	-	150	150	80	80
Pea protein‡	-	-	-	-	80	80
Glutalys®‡	-	-	-	-	60	60
Viten Wheat gluten®‡	-	-	-	-	60	60
Fish oilδ	121	121	157.1	157.1	154.2	154.2
Corn starch§	183.4	183.4	19.5	19.5	101.1	101.1
Vitamin-mineral premixʒ	20	20	20	20	20	20
CMC-binder§	5	5	5	5	5	5
Bactocell®*	-	0.02	-	0.02		0.02
Proximate composition (%)						
Moisture (%)	3.9 ± 0.3	2.5 ± 0.2	2.5 ± 0.8	2.3 ± 0.2	3.1 ± 0.7	1.9 ± 0.4
Solids (%)	96.1 ± 0.3	97.5 ± 0.2	97.5 ± 0.8	97.7 ± 0.2	96.9 ± 0.7	98.1 ± 0.4
Crude protein (%)	50.8 ± 1.4	51.8 ± 1.1	50.8 ± 0.8	50.0 ± 1.7	50.8 ± 0.8	49.0 ± 0.4
Lipids (%)	19.4 ± 1.5	20.6 ± 4.4	19.3 ± 0.3	20.0 ± 1.0	20.7 ± 1.8	18.8 ± 0.6
Ash (%)	9.3 ± 0.2	9.4 ± 0.2	7.5 ± 0.3	7.6 ± 0.1	6.7 ± 0.3	5.0 ± 1.0
Gross energy (MJ kg^{-1})	21.9 ± 0.2	22.1 ± 0.1	22.1 ± 0.0	21.7 ± 0.1	22.7 ± 0.1	22.1 ± 0.0

Proximate composition data are mean ± SD, n = 3.
ϕ Herring meal LT94 – United Fish Products Ltd., Aberdeen, UK.
¥ BioMar.
‡ Roquette Frêres, France.
δ Epanoil, Seven Seas Ltd, UK.
§ Sigma, UK.
ʒ Premier Nutrition vitamin/mineral premix: 121 g kg-1 calcium, Vit A 1.0 µg kg-1, Vit D3 0.1 µg kg-1, Vit E (as alpha tocopherol acetate) 7.0 g kg-1, Copper (as cupric sulphate) 250 mg kg-1, Magnesium 15.6 g kg-1, Phosphorous 5.2 g kg-1.
**Pediococcus acidilactici* (CNCM MA 18/5 M), Bactocell® (Lallemand Inc., Canada).

3.3.3. Diet preparation

The dry ingredients were weighed and mixed thoroughly for approximately 1h using a Hobart food mixer (Hobart Food Equipment model no: HL1400–10STDA, Australia). Thereafter, the fish oil was gradually added to the ingredients. After further mixing, warm water was added to produce a soft and slightly moist consistency suitable for cold press extrusion. Control diets were prepared before the probiotic diets to avoid cross contamination. For the probiotic diets, 200 mg of Bactocell® was first mixed with corn starch using a commercial blender to produce a well-homogenised mixture. This mixture was added gradually to the remaining ingredients. After mixing all the ingredients, the mixture was passed through a pelleter (PTM P6 extruder, Plymouth, UK) to produce 4 mm pellets which were then spread out and dried using an air convection oven at 45 °C for 36h. After drying, the diets were broken up to the appropriate size and stored in airtight containers at 4 °C until use. New batches of diets were produced every four weeks to ensure that the viability of the probiotic bacterium was maintained for the duration of the trial. The viability of *P. acidilactici* in the probiotic diets (displayed in Figure 3.1) was determined by plate counts on MRS (de Man, Rogosa and Sharpe) agar incubated aerobically at 37 °C for 48h and then verified by PCR and 16rRNA gene sequencing as described by (Ferguson et al., 2010).

3.3.4. Sample collection

After twelve weeks of feeding, three fish per tank were euthanised and sampled. The intestine was aseptically removed using sterile instruments and divided into the proximal and distal intestine. Distal intestinal digesta was pooled by tank and collected into a sterile tube. Fish dissection and sampling methodology are described in Section 2.3.

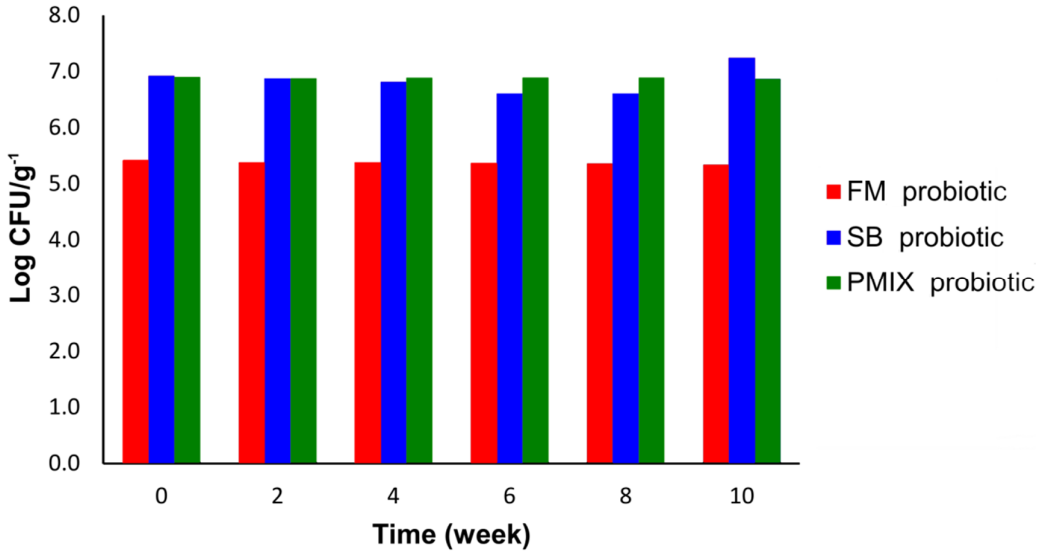


Figure 3.1. The viability of *P. acidilactici* in each basal diet during 10 weeks. Fishmeal (FM), SB (soybean) and PMIX (plant mix).

3.3.5. Growth performance

Increase in weight gain (WG), specific growth rate (SGR), and feed conversion rate (FCR) were calculated as described in Section 2.2.

3.3.6. Microbiological analysis

For analysis of the distal intestinal microbiota, digesta from three fish were sampled and pooled by tank (n = 3), sampling was conducted as described in Section 2.3. and the analysis was conducted according to Section 2.4. Digesta samples were pooled per tank (3 samples per treatment).

3.3.7. Statistical analysis

Statistical comparisons in all the analyses were conducted between control and probiotic group in the same basal diet, and also among basal diets using only the control treatments. Statistical analysis was carried out using the methods described in Section 2.5.

3.4. Results

3.4.1. Growth performance

Growth performance, including weight gain (WG), specific growth rate (SGR) and feed conversion ratio (FCR) showed similar trends in all the experimental groups at week 12. Therefore, only SGR results are described in this section. The results of SGR from week four to week twelve are displayed in Figure 3.2. No significant differences were evident in growth performance between treatments (control and probiotic groups) in any of the diets. Fish fed the FM diet had a significantly higher (P > 0.05) SGR regardless of the treatment compared with the fish fed the PMIX and SB diets, whereas no significant differences in SGR were seen between fish fed PMIX and SB diets.

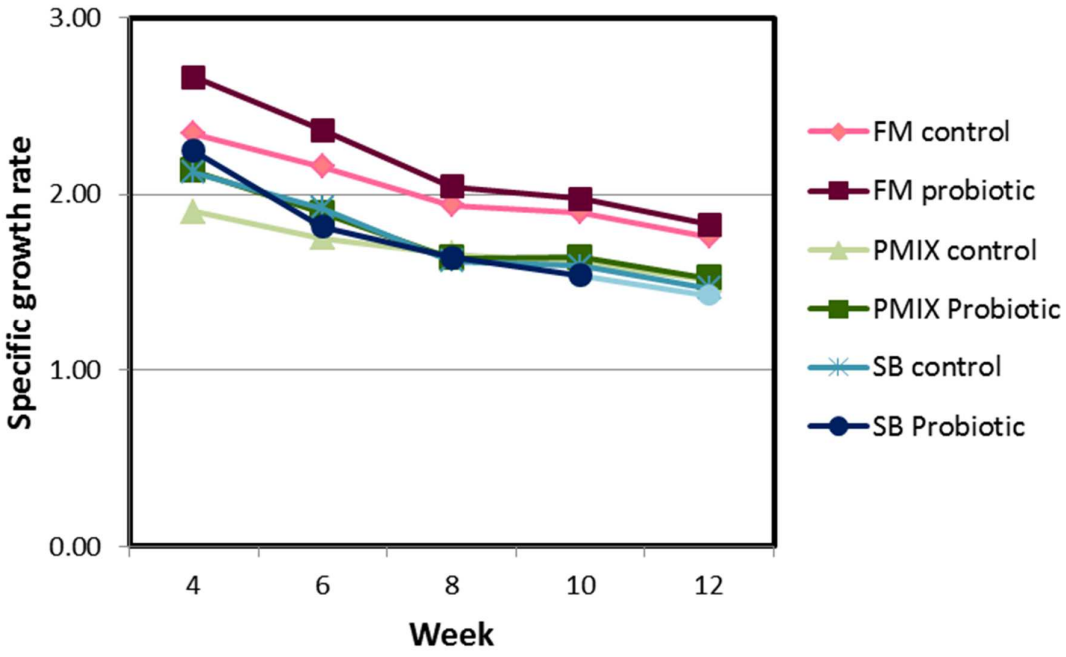


Figure 3.2. Specific growth rate from week four to week twelve of fish fed control and probiotic basal diets.

3.4.2. Microbiota analysis

3.4.2.1. High-throughput sequencing data

Eighteen samples from distal intestinal digesta were processed on the Ion Torrent platform to analyse the bacterial microbiota associated with the digesta of the distal intestine. High-throughput sequencing (HTS) generated 3,617,063 reads before quality control (200,948 ± 46,414 reads per sample). After quality filtering, processing the data in QIIME, filtering spurious sequences and discarding reads belonging to Streptophyta a total of 2,020,429 reads remained (112,246 ± 33,127 reads per sample). Reads belonging to Streptophyta were significantly lower in the fish fed the FM diet (281 ± 109) in comparison with fish fed the SB (43217 ± 31729), or PMIX (5620 ± 1922) diets regardless of the supplementation of probiotic in the diet.

3.4.2.2. Intestinal microbiota in the digesta of distal intestine

The results presented in this section will focus on the differences between control and probiotic groups within each diet as well as the differences in the microbial communities across the control groups of all the diets.

Alpha diversity parameters such as Chao1, phylogenetic diversity (PD) and Shannon index revealed significant differences between the control and probiotic groups in the fish fed the SB diet (Figure 3.3.). The control groups among different diets had significant differences in the Shannon diversity index. Meanwhile, differences in control groups using PD parameters were only significant between PMIX and SB groups.

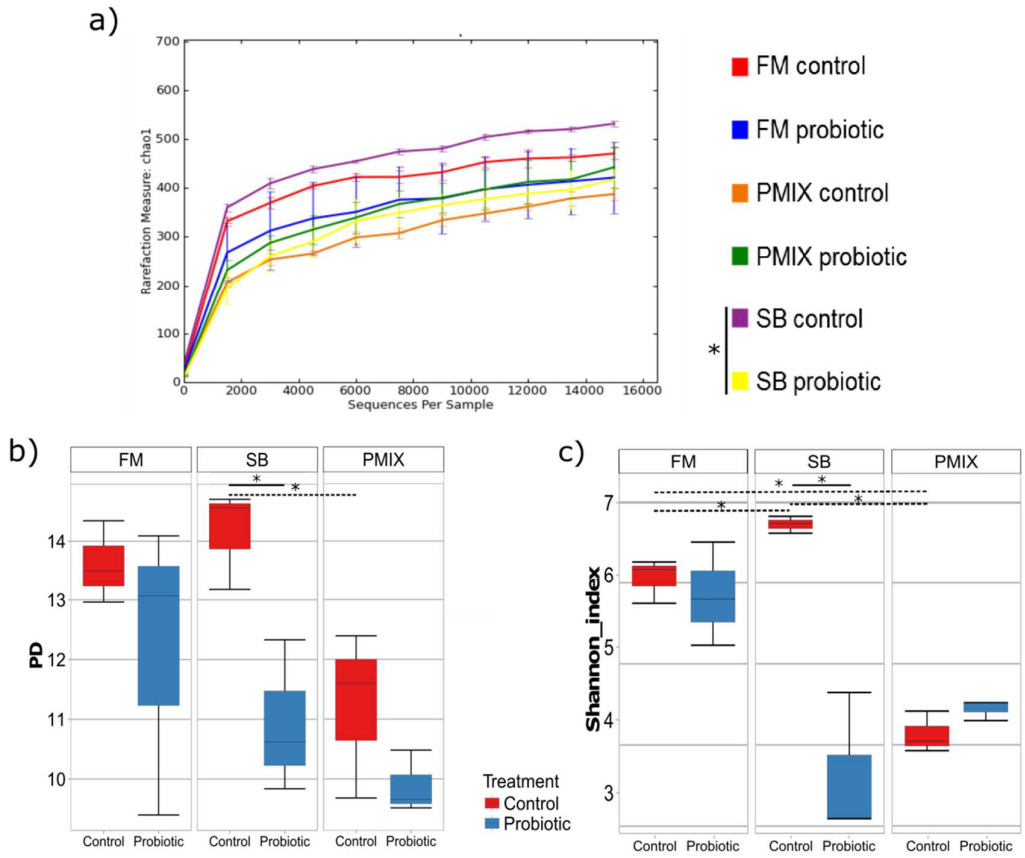


Figure 3.3. Alpha diversity parameters of the distal intestinal microbiota comparing probiotic and control groups among fishmeal (FM), soybean (SB) and plant mix (PMIX) diets. a) Rarefaction curve based on Chao1 metric representing the average and standard deviation (error bars) of OTUs per experimental group; b) Phylogenetic diversity (PD) boxplot; c) Shannon index boxplot. Statistical analysis was conducted using alpha diversity parameters from samples rarefied at an even depth of 15,000 sequences. Statistical differences are denoted by asterisk * ($P < 0.05$). Significant differences between control (red) and probiotic (blue) within the same diet are denoted by solid lines, whilst dashed lines represent significant differences between control groups of different diets.

Beta diversity based on weighted and unweighted UniFrac dissimilarity matrix was used to compare differences in the bacterial composition between treatment and basal diets, and the results are visualised in a principal coordinate analysis (PCoA) (Figure 3.4). The principal coordinate analysis showed that the primary factor influencing the cluster of differentiation among the experimental groups was the diet for both weighted and unweighted UniFrac. A lower clustering effect was observed between treatments (i.e. control and probiotic groups). Clustering between treatments was more evident in

the PCoA from unweighted UniFrac than weighted UniFrac in SB and PMIX groups. The latter clustering effect by diet factor was also demonstrated in the highly significant differences and Pseudo-F shown by PERMANOVA analysis (Pseudo-F 24.05, *P* = 0.001) and (Pseudo-F 4.49, *P* = 0.001) for weighted and unweighted UniFrac, respectively. The highest differences according to the weighted UniFrac in the bacterial composition of the diets were seen between FM vs. PMIX diets (Pseudo-F, 6.57), followed by SB vs. PMIX (Pseudo-F, 5.90). PERMANOVA analysis also revealed a significant interaction between diet and treatment factors. According to pair-wise comparison based on weighted UniFrac, the greatest difference between treatments was in the fish fed the SB diet (t, 3.15). In contrast, only minor differences in pair-wise comparison among treatments were observed in the unweighted UniFrac.

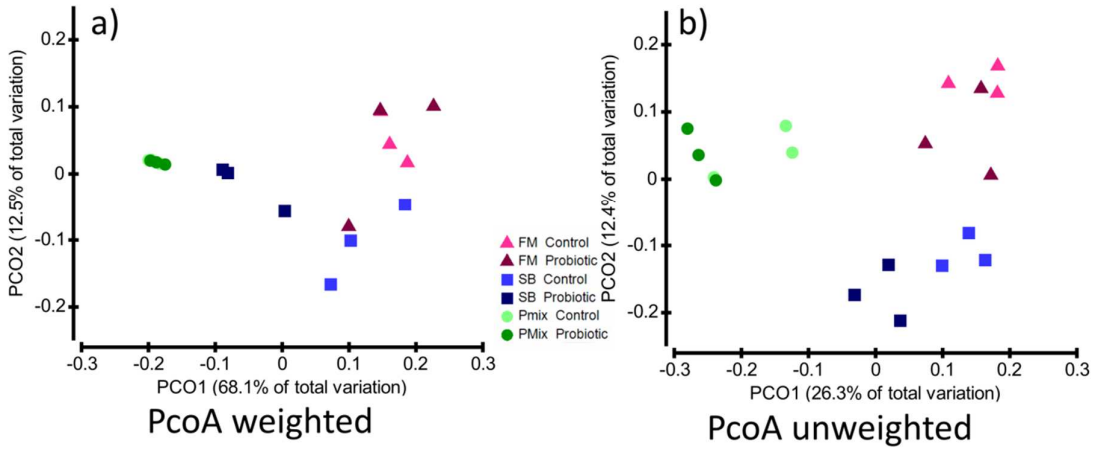


Figure 3.4. Principal coordinate analysis (PCoA) of the distal intestinal microbiota associated to digesta using UniFrac distances. The percentage of variation is explained by PC1 and PC2 axis. Each plot represents the differences among the different basal diets. Fishmeal (FM, triangles), soybean (SB, squares) and plant mix (PMIX, circles) as well the differences between control (lighter colours) and probiotic (dark colours) groups differences a) PCoA weighted digesta; b) PCoA unweighted digesta.

Tabla 3.2. PERMANOVA results from weighted and unweighted UniFrac.

Factor/Group comparison	PERMANOVA					
	Weighted UniFrac			Unweighted UniFrac		
	Average dissimilarity	Pseudo-F/t	*P*	Average dissimilarity	Pseudo-F/t	*P*
Diet		24.05	0.001		4.49	0.001
Pair-wise for Diet					2.20	0.002
FM vs SB	0.25	2.67	0.003	0.46	1.69	0.006
FM vs PMIX	0.37	6.57	0.002	0.52	2.36	0.001
SB vs PMIX	0.27	5.90	0.004	0.49	2.26	0.008
Treatment		5.11	0.001		2.20	0.002
Diet x Treatment		3.63	0.003		1.19	0.143
Pair-wise for Treatment						
FM-Control vs Probiotic	0.16	0.90	0.709	0.43	1.17	0.208
SB-Control vs Probiotic	0.27	3.15	0.101	0.40	1.28	0.106
PMIX-Control vs Probiotic	0.04	1.50	0.104	0.39	1.26	0.116

Fishmeal - (FM); soybean - (SB); plant mix - (PMIX).

Figure 3.5. shows the relative abundance of OTUs from pooled digesta samples (per tank) for each experimental group at class and phylum levels. All experimental groups were dominated by six phyla, which accounted for more than 95% of the total relative abundance. The dominant taxa belonged to the phylum Firmicutes, mainly classes Bacilli and Clostridia, which together accounted for more than 75% of the total reads abundance for the FM and SB groups. In the PMIX group, more than the 90% of the total reads belonged to the class Bacilli. Proteobacteria, Actinobacteria and Fusobacteria were also important taxa in terms of abundance in FM and SB groups, but not in the PMIX group. At genus level, the most dominant taxa varied according to the diet and treatment. In the FM group, the most abundant genera were *Peptostreptococcus* (25.1% ± 13) and an unidentified genus from the order Clostridiales (12.1% ± 6) in both control and probiotic treatments. Meanwhile, *Lactobacillus* (48.7% ± 8) and *Bacillus* (33.3% ± 5) were the most abundant genera in the PMIX group. Finally, the dominant taxa in the SB group varied according to the treatment. An unidentified genus from family *Leuconostocaceae* (11% ± 8) and *Peptostreptococcus* (10.7% ± 8) were the most abundant taxa in the control group;

meanwhile among the indentified genera *Pediococcus* was the dominant taxon in the treatment supplemented with the probiotic.

The genus *Pediococcus* was detected in all the experimental groups with important differences according to the diet and treatments factors. In overall, the control groups for all the three different basal diets had a low abundance of this genus ranging from 0.06% to 0.38%. On the other hand, *Pediococcus* abundance varied greatly in the treatment groups among the different diets. The highest abundance of *Pediococcus* was seen in the treatment group fed the SB diet (83.5% ± 15). Meanwhile, the *Pediococcus* abundance in the treatment groups fed the FM and PMIX were 15.4% ± 11 and 5.9% ± 3, respectively.

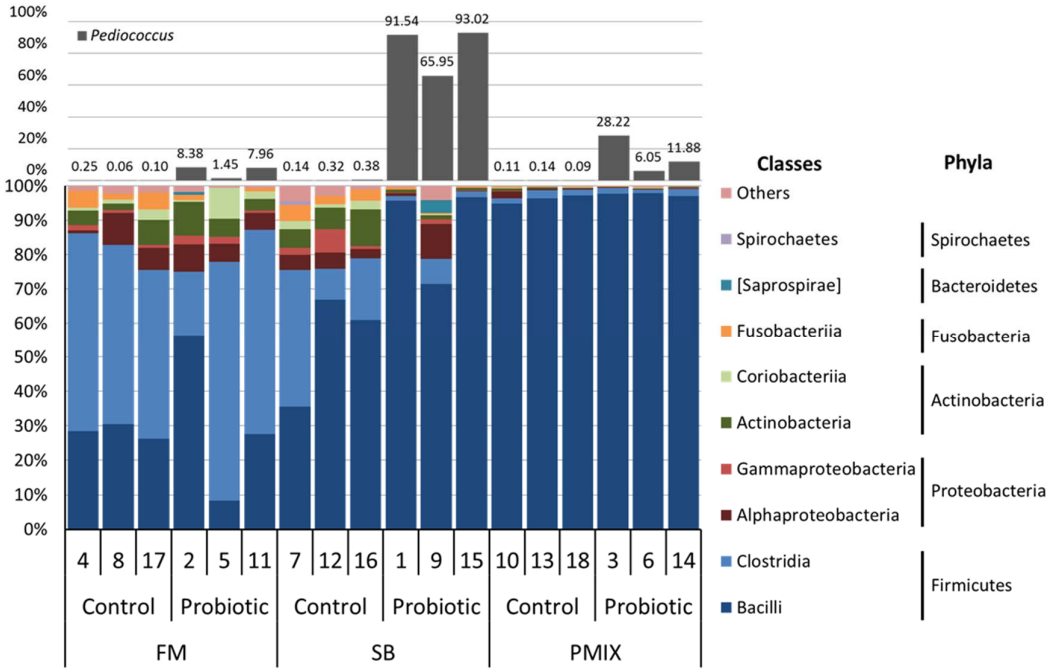


Figure 3.5. Relative abundance of the main bacterial taxa at class and phylum level and abundance of the genus *Pediococcus*. Classes below an abundance average of 0.8% per experimental group are not shown but summarised in a mixed group "Other". Numbers below the bars represent the number of the tank for each experimental group. Fishmeal (FM), soybean (SB) and plant mix (PMIX).

Significant differences in specific taxa between treatments and among the different basal diets were analysed with Linear discriminant analysis effect size (LEfSe) (Figure 3.6.). Overall, the greatest differences between control and probiotic were found in the group fed the SB diet with 46 genera significantly modulated, compared with the group fed the FM and PMIX diets that had 12 and 13 genera significantly modulated, respectively. Most of the taxa modulated by treatment resulted in the enrichment of such taxa in the control group and only a few taxa were enriched in the probiotics groups regardless the basal diet.

Regarding the fish fed the FM diet, the main differences between the control and probiotic groups were the enrichment of the genera *Fusobacterium* and *Bacteroides* in the control group. Other taxa that were also enriched in the control group, although to a lesser extent, belonged to the class Gammaproteobacteria (genera *Pseudomonas* and *Vibrio*) and phylum Firmicutes (genera *Ruminococcus*, *Blautia* and *Kuthia*). The only enriched taxa in the treatment group fed the FM basal diet were the taxa belonging to the genera *Pediococcus* and *Sphingomonas*. In the fish fed the SB diet, the main taxa significantly enriched in the control group belonged to the Firmicutes phylum (classes Clostridia and Bacilli), followed by Actinobacteria (classes Actinobacteria and Coriobacteriia) and Fusobacteria (genus *Cetobacterium*). The genus *Bacteroides* and several genera belonging to the phylum Proteobacteria were also enriched in the control group of the SB diet. The main differentially modulated taxa in the fish fed the PMIX diet belonged to Proteobacteria (genus *Vibrio*), Actinobacteria (genera *Arthrobacter* and *Brevibacterium*) and Firmicutes phyla (genera *Enterococcus* and *Tepidimicrobium*). All these taxa were enriched in the control group. To evaluate the sole effect of the basal diet on the bacterial microbiota associated to digesta, the high-throughput sequencing data from the control groups of

the three basal diets were compared with LEfSe (Figure 3.7.). The greatest number of overrepresented genera was observed in the fish group fed the FM diet (13 genera enriched), followed by SB and PMIX diets with six and one genera enriched, respectively. The main differences between fish fed the basal diets were observed in the phylum Firmicutes (classes Clostridia and Bacilli). The class Clostridia was significantly overrepresented in the FM group compared with the two diets using plant ingredients. In the SB diet group, there was an enrichment of the phyla Cyanobacteria as well as some taxa from the Proteobacteria (genera *Mycoplana*, *Pseudomonas*) and Firmicutes phyla (genus *Facklamia*, unidentified members of the family *Leuconostocaceae* and the order Bacillales).

The shared OTUs at tank level were determined for each of the three different basal diets including both treatments (Figure 3.8.). In this study, the core microbiota was defined as the shared OTUs among the 80% of the samples for each experimental group. The core microbiota was comprised by 66 OTUs, which represented 52% of the shared OTUs for each dietary group evaluated. The OTUs in the core microbiota belonged to three phyla, Actinobacteria, Firmicutes and Proteobacteria. The phylum with the highest number of OTUs belonging to the core was Firmicutes, which had 37 OTUs divided into five different orders. The most represented orders were Lactobacilliales with 14 OTUs followed by Clostridiales with 12 OTUs. These two orders were also the most important regarding average abundance in all the experimental groups except in the PMIX group where the orders Lactobacilliales and Bacilliales were the most abundant. In general terms, the contribution in abundance of the set of taxa that composed the core microbiota was high in all the experimental groups, ranging from 69.1% to 96.8%.

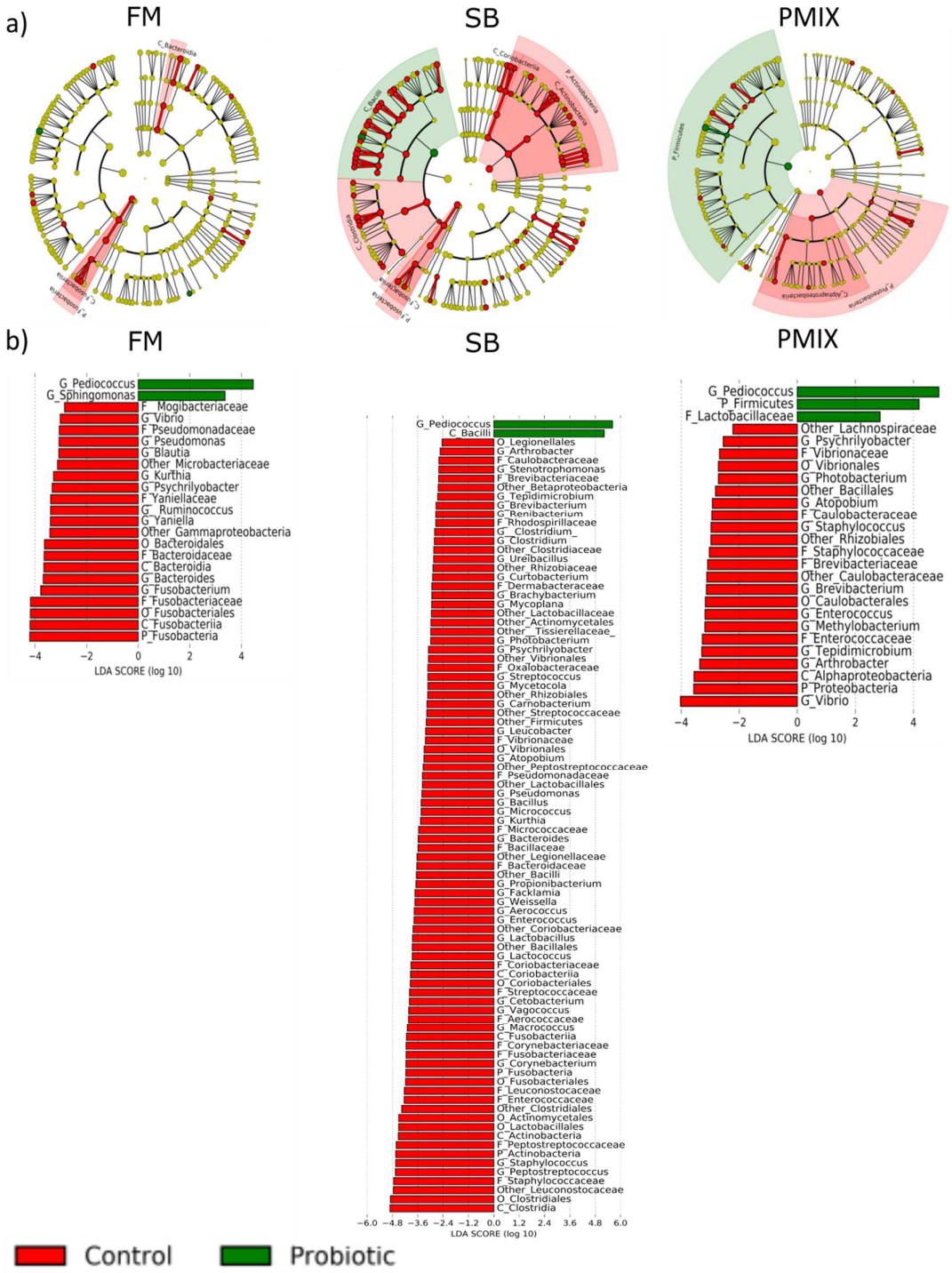


Figure 3.6. Taxonomic differences in the distal intestinal microbiota associated to digesta between control and probiotic groups according to LEfSe analysis. The analysis was carried out with the relative abundance of all the samples at the genus level. Control and probiotic groups were treated as classes. a) A circular cladogram is representing the significant enriched OTUs between control (red) or probiotic (green) groups. No significantly different OTUs are represented in yellow. The diameter of each dot is proportional to its effect size. b) Linear discriminant analysis (LDA), differentially enriched OTUs are arranged in descending order according to LDA score. Fishmeal (FM), soybean (SB) and plant mix (PMIX).

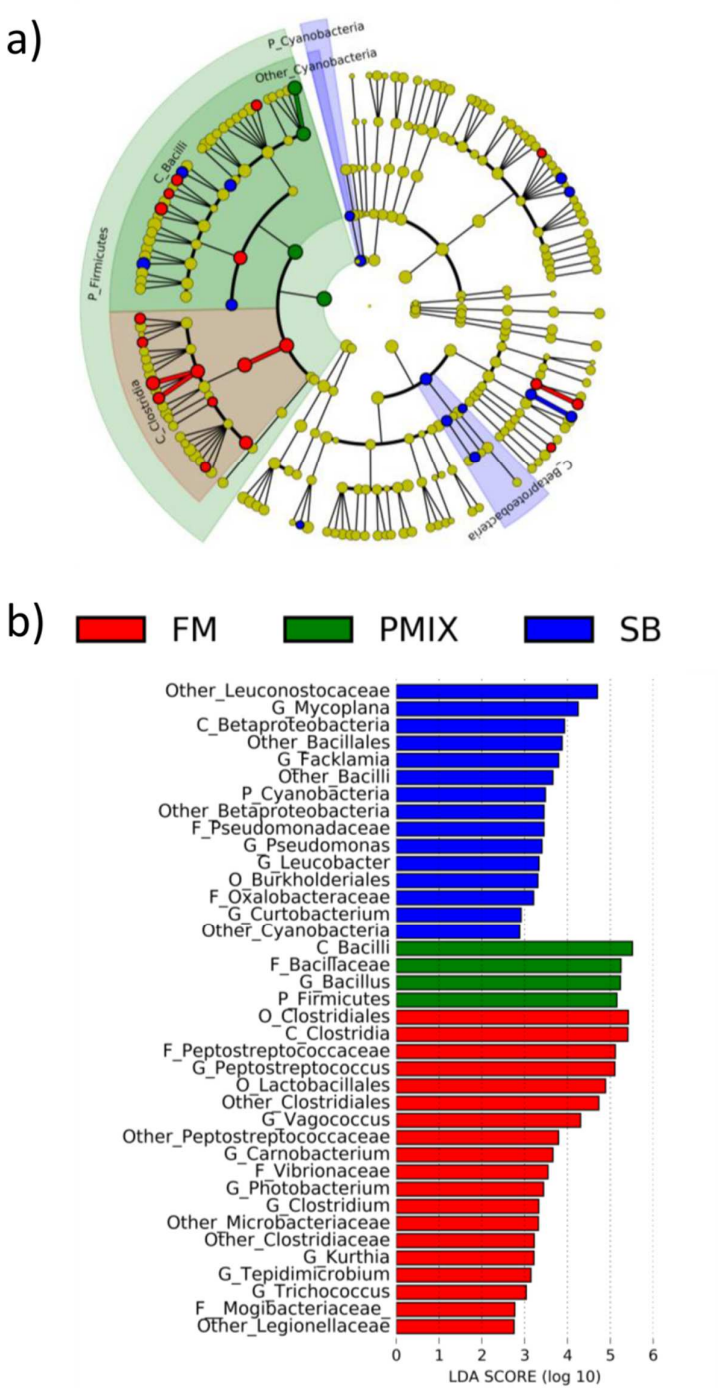
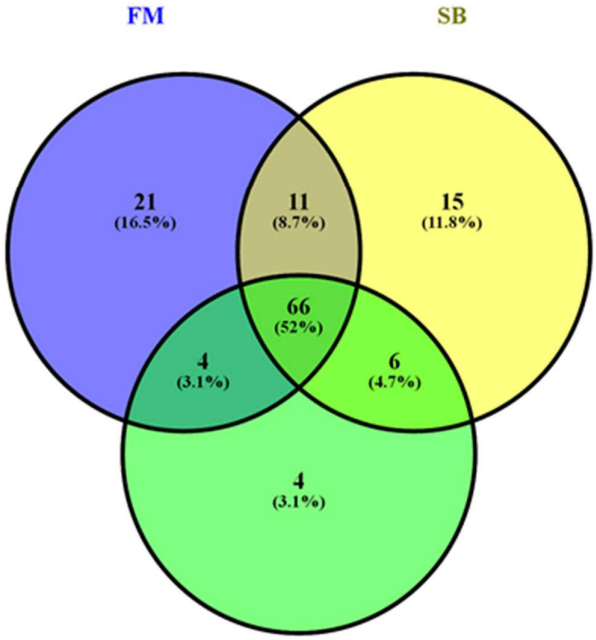


Figure 3.7. Taxonomic differences in the distal intestinal microbiota among dietary groups according to LEfSe analysis. The analysis was carried out with the relative abundance of all the control samples at the genus level. FM, SB and PMIX were treated as classes. a) A circular cladogram is representing the significant enriched OTUs between FM (red) PMIX (green) and SB (blue) groups. No significant different OTUs are represented in yellow. The diameter of each dot is proportional to its effect size. b) Linear discriminant analysis (LDA), differentially enriched OTUs are arranged in descending order according to LDA score. Fishmeal (FM), soybean (SB) and plant mix (PMIX).

a)



b)

Core Taxonomy		Number of OTUs in the order	Average abundance (%)					
Phylum	Order		FM-C	FM-P	SB-C	SB-P	PMIX-C	PMIX-P
Actinobacteria	Actinomycetales	10	3.04	4.46	6.10	0.69	0.31	0.19
	Coriobacteriales	2	1.73	3.90	1.94	0.17	0.14	0.05
Firmicutes	Unidentified Firmicutes	1	0.03	0.08	0.01	0.01	0.16	0.13
	Bacilliales	7	0.94	1.12	14.74	1.07	36.09	31.21
	Lactobacilliales	14	11.12	18.86	15.84	85.34	56.82	63.35
	Clostridiales	12	49.57	48.25	21.49	3.38	1.83	1.49
Bacteroidetes	Bacteroidales	1	1.06	0.03	1.16	0.32	0.04	0.08
Fusobacteria	Fusobacteriales	3	3.42	0.71	3.35	0.33	0.22	0.10
Proteobacteria	Unidentified Alphaproteobacteria	1	0.93	0.12	0.01	0.00	0.17	0.00
	Caulobacterales	2	1.29	3.17	2.09	0.77	0.30	0.04
	Rhizobiales	5	2.81	2.09	0.98	1.02	0.29	0.08
	Unidentified Betaproteobacteria	1	0.01	0.01	0.02	0.00	0.00	0.00
	Burkholderiales	2	0.12	0.27	0.40	0.07	0.03	0.04
	Enterobacteriales	1	0.01	0.00	0.51	0.03	0.01	0.00
	Pseudomonadales	1	0.12	0.02	0.16	0.07	0.00	0.01
	Vibrionales	1	0.59	1.04	0.27	0.09	0.04	0.01
	Xanthomonadales	1	0.04	0.04	0.08	0.00	0.02	0.00
	Brevinematales	1	0.30	0.00	0.01	0.63	0.00	0.00
	Total	66	77.13	84.19	69.15	93.99	96.46	96.80

Figure 3.8. Core microbiota of distal intestinal digesta. a) Venn diagram showing the shared OTUs across 80% of the samples per diet (fishmeal (FM), soybean (SB) and plant mix (PMIX)) including control and probiotic groups. b) Table showing the contribution of each component (average abundance) of the core microbiota in each experimental group (fishmeal control (FM-C), fishmeal probiotic (FM-P), soybean control (SB-C), soybean probiotic (SB-P), plant mix control (PMIX-C), plant mix probiotic (PMIX-P).

3.5. Discussion

A major challenge that the salmonid industry is currently facing is the need for sustainable feed ingredients that decrease the dependence on marine ingredients such as fishmeal without affecting the fish health and the quality of the final product (Naylor and Burke, 2005; Gatlin et al., 2007; Hardy, 2010; Ytrestøyl et al., 2015). In the same line, the use of functional feeds, which incorporate probiotics, has gained attention due to their potential to improve fish health. Successful use of a probiotic to improve fish health depends on a broad range of factors. One central factor that can interact negatively or positively with the probiotic is the diet composition. This is particularly important when the probiotic is supplied in the diet. Thus, it is fundamental to study the potential interaction between novel feed ingredients and probiotics, to accomplishing possible benefits of both on the fish. The present study evaluated the impact of different diets using a high content of plant ingredients to replace the fishmeal on the distal microbiota and their interaction with a dietary supplementation of a commercial probiotic in rainbow trout. Results showed that the diet with high content of plants and the probiotic bacterium *P. acidilactici* modulated the intestinal microbiota in the digesta of the distal intestine and this effect was dependent on the diet used.

3.5.1. Effect of probiotic supplementation and fishmeal replacement in growth performance of rainbow trout.

In this study, the growth performance of fish fed the experimental diets was investigated. As it was expected, the fish fed the FM diet had better growth performance than fish fed the SB and PMIX diets. The inclusion of plant ingredients to replace FM in the SB and PMIX diets was 62%. This level of replacement is considered

high for salmonids and leads to one possible explanation for the lower growth in both groups of fish fed the plant-based diets in comparison with the fish fed the FM diet. Plant ingredients based on soybean and pea protein as the ones used in this study are known to have antinutritional factors (Krogdahl et al., 2010), which have detrimental effects on fish health and growth performance in salmonids. No significant differences were found between fish fed the probiotic or control diets in any of the basal diets. The previous reports indicating the potential of supplementation of *P. acidilactici* in the diet on increasing the growth performance in salmonids is contradictory. Three studies in rainbow trout studying the effect of *P. acidilactici* as single-species probiotic in growth performance did not observe differences between fish fed probiotic diet in comparison with the control group (Aubin et al., 2005; Merrifield et al., 2011; Hoseinifar et al., 2015a). In contrast, Ramos et al. (2015) reported an increase in feed conversion of rainbow trout fed a diet supplemented with high doses of *P. acidilactici*. In this study, the authors suggested that an increase in feed conversion was dose dependent, as this effect was not observed in the fish fed low doses of *P. acidilactici*.

3.5.2. High-throughput sequencing based on 16S rRNA gene of intestinal samples of fish fed plant ingredients is affected by producing large number of non-bacterial sequences

Several studies using high-throughput sequencing to study intestinal microbiota in trout have been published in the last years using different platforms and pipelines (Desai et al., 2012; Wong et al., 2013; Ingerslev et al., 2014b). In this study, the sequencing of bacterial DNA using the so-call “universal primers” for the V1-V2 region of the 16S rRNA gene in the Ion Torrent platform demonstrated to be reliable to

capture the diversity of the intestinal microbiota of rainbow trout. The latter is supported by the results from alpha diversity suggesting that the depth of sequencing was adequate to detect the diversity of all the samples analysed. Although a high number of reads were retained after bioinformatics processing, one interesting finding was a large number of reads eliminated after filtering out the reads affiliated to Streptophyta, which are commonly associated with chloroplast contamination from plants. The number of reads discarded has been particularly high in the samples from the experimental groups from SB and PMIX diet. The latter corroborated that the presence of Streptophyta in the intestine of fish is associated to the presence of plant contents (chloroplast) and its detection in the bioinformatics data is probably due to the similarities in the chloroplast sequences and the bacterial 16S sRNA genes (Hanshew et al., 2013). Previous studies in fish, have also detected this issue and most of the authors have chosen to remove the reads belonging to Streptophyta or chloroplast-related sequences from the analysis, arguing that these reads are contamination or artefacts (Estruch et al., 2015; Eichmiller et al., 2016; Zarkasi et al., 2016). Meanwhile, other authors have included Streptophyta in their analysis assuming that is part of the bacterial communities of the intestine (Ingerslev et al., 2014b; Parma et al., 2016). High homology between bacterial and chloroplast 16S rRNA gene could lead to discarding a high number of sequences resulting in data bias, additional resources and time to increase sequencing depth to improve the alpha diversity analysis. In aquaculture, difficulties in differentiating true bacterial sequences from plant contamination could become a considerable limitation for studying the gut microbiota of herviborous fish or fish fed diets with high inclusion of plant ingredients. Some alternatives methodologies include the use of primers targeting genes other

than 16S rRNA and alternative DNA extraction protocols should be taken into account in future studies to overcome these limitations.

3.5.3. Probiotic supplementation in the diet interacts with diet composition modulation; the role of probiotic on intestinal microbiota.

Microorganisms with the potential to be used as dietary supplements should be recovered in acceptable, viable concentrations after feed processing and subsequent storage. In this study, one interesting finding regarding the viability evaluation was the lower recovery of *P. acidilactici* in the FM basal diet in comparison with the diets with a high content of plant material, i.e., SB and PMIX diets. Despite the fact, that the inclusion of the commercial product was the same in all the basal diets (200 mg per kg), the FM diet had more than ten times lower concentration of *P. acidilactici* that the other two diets. Although the cause of this is unknown, this observation may support the hypothesis that specific compounds in fishmeal diet or any of the plant ingredients used in SB and PMIX diets inhibit or favour the survival of *P. acidilactici*. On the other hand, even though the viability of *P. acidilactici* in both plant-based diets was similar after feed processing, a significant difference in the relative abundance of *Pediococcus* according to high-throughput sequencing was observed between fish fed the SB and the PMIX diets. These results suggest that specific components in the plant-based diet could not only affect the survival of this bacterium during the feed processing but also promote or decrease the survival of *P. acidilactici* during transit in the gastrointestinal tract of the fish. These results are relevant since the number of viable cells of the probiotic in the intestine could be related to the extent of the effect in the host (reviewed by Merrifield et al. (2010c)).

Results in the present study indicated a significant interaction between treatment and diet factors detected in the PERMANOVA analysis. Moreover, alpha diversity and LEfSe showed that the intestinal microbiota is modulated in a greater extent in the group where a higher abundance of *Pedioccocus* was found, i.e., fish fed the SB diet in comparison with the fish fed the PMIX and FM groups. LEfSe analysis indicated that only one OTU was consistently modulated by the dietary supplementation across the three experimental groups fed the basal diets. This OTU is an obligate anaerobic bacterium and recently recognised genus, i.e., *Psychrilyobacter* belonging to the phylum Fusobacteria (Zhao et al., 2009). Although little information is found in the literature about this genus, it has been reported as a normal inhabitant of the intestinal microbiota of rainbow trout and parr Atlantic salmon (Ingerslev et al., 2014a; Dehler et al.). Taken together, these findings demonstrated that the effect of probiotic on the intestinal microbiota was dependent on the basal diet as seen by the similarities in some of the differentially modulated OTUs between the groups fed the diets containing plant ingredients in contrast with groups fed the diets containing fishmeal as the main protein source.

To the author's knowledge, the impact of the diet on the role of probiotic on the microbial communities in the intestine of fish has not been comprehensively studied. Only two reports are known in evaluating the effect of a probiotic supplementation with *P. acidilactici* and different diet formulations in rainbow trout (Ingerslev et al., 2014a; Ingerslev et al., 2014b). These authors reported that diet ingredients were the main factor modulating the intestinal microbiota, whereas non-effect of probiotic supplementation on intestinal microbiota was detected. Some similarities between the present study and the previous reports showing that the bacterial communities in the

rainbow trout are modulated when the fishmeal is replaced by a plant protein such as pea protein.

3.5.4. Feed ingredients in the diet influence the intestinal microbiota

Factors that modulate the gut microbiota such as diet and probiotic supplementation have been previously studied in rainbow trout (reviewed by Romero et al. (2014)). In the present study, to assess only the effect of the diet factor on intestinal microbiota, the control samples from the three different basal diets were compared. Consistently with the findings in alpha and beta diversity, the present study revealed that the diet is a major factor that modulated the intestinal microbiota. However, the extent of modulation varied according to diet. Surprisingly, substantial differences in the overall bacterial community structures according to beta diversity results were observed between fish fed both plant-based diets. The dissimilarity in the intestinal microbiota between fish fed the plant-based diets was higher than between the fish fed the SB and the FM diets. This finding was unexpected as both plant-based diets are more similar between them regarding the ingredients used in comparison to the fishmeal diet. Moreover, the lowest alpha diversity was detected in fish fed the PMIX diet; meanwhile, the highest alpha diversity was observed in the fish fed the SB diet followed by the fish fed the FM diet. These results may suggest that the alpha diversity results had the same pattern in the beta diversity results with substantial differences between both plant diets. Other authors have reported important differences in the bacterial communities of fish fed mainly marine ingredients in contrast with fish fed high inclusion of plant ingredients. Partial replacing of fishmeal by soybean and other plant ingredients in salmonids has been associated with alteration of intestinal microbiota (Desai et al., 2012; Ingerslev et al., 2014b; Reveco et al., 2014). In the

present study, SB and PMIX diets also induced significant changes in the intestinal microbiota compared with the FM diet. Some authors have reported that inclusion of plant ingredients in the diet led to an increased abundance of members of the Firmicutes phylum (Desai et al., 2012; Ingerslev et al., 2014b). On the other hand, studies conducted by Wong et al. (2013) showed that bacterial communities in the intestine of rainbow trout were not affected by total replacement of fishmeal and blood meal by soy protein concentrate and corn gluten meal. The results of the present study observed a significant increase of the Firmicutes phylum but only in fish fed the PMIX diet. This change was led by the high proportion of genus *Bacillus* found in this group compared to fish fed the SB and FM diets. Interestingly, a significant shift within the phylum Firmicutes was observed in the class Clostridia which was highly abundant in the fish fed FM diet and had decreased in plant-based diets especially in the fish fed the PMIX. These results differ from previous studies evaluating replacing of fishmeal by plant ingredients which did not report an increase of the class Clostridia in fish fed diets based on fishmeal (Desai et al., 2012; Ingerslev et al., 2014b). Instead, the latter studies observed a shift in the intestinal microbiota represented by an increase of Proteobacteria and decrease of Firmicutes, which was not noted in the present study. Regarding the phylum Proteobacteria, the relative abundance of this phylum in the present study was lower in fish fed the FM diets in comparison with previous reports. These differences between studies could be the result of different environmental conditions, methodologies, aquarium facilities and experimental design used in these studies.

3.5.5. Core microbiota and main phyla and taxa found in this study compared with previous studies in rainbow trout

The term core microbiota has been used to define the set of dominant and stable microbial species in a specific host habitat such as the gut (Salonen et al., 2012). However, no agreement has been achieved to standardise the threshold to define the core microbiota. In this study the threshold of core microbiota was defined as the set of common OTUs at genus level in the 80% of the samples. The results indicating a high number of OTUs (66) as core microbiota is unexpected as there were two factors i.e., diet and treatment that influenced the microbiota. This finding is in line with Wong et al. (2013) who investigated the effect of two different factors i.e., diet and rearing density in the core microbiota of rainbow trout. These authors identified 52 OTUs shared in all the samples and concluded that rainbow trout has a large core microbiota resistant to variation in diet and rearing density. In order to provide a more comprehensive analysis of the core microbiota, a comparison of the core microbiota between the previous study from Wong et al. (2013) and the results of the present study was performed. The results of this comparison detected 32 OTUs in common at genus level. These OTUs belonged to the phyla Firmicutes (Orders Lactobacilliales, Clostridiales and Bacilliales) and Betaproteobacteria (Orders Caulobacterales Rhizobiales, Burkholderiales, Enterobacteriales and Pseudomonadales). Moreover, in both studies all taxa belonging to the core microbiota account for more than the 80% of the total average abundance of each experimental group. The similarities between members of the core microbiota in both studies and their relative dominance in the intestinal microbiota suggest that these taxa have an important role in the intestine of rainbow trout. Nonetheless, although some similarities were found with the study conducted by Wong et al. (2013), it is important to highlight that the core microbiota in

the present study had important changes among the experimental groups fed the control basal diet. Thus, remarkable differences in the phylum Firmicutes, specifically the orders Bacilliales, Lactobacilliales and Clostridiales were found in terms of relative abundance among groups fed with the different basal diets.

3.5.6. Conclusion

In conclusion, this study revealed that specific ingredients in the diet formulation are able to modulate the effect of a commercial probiotic on the intestinal microbiota of rainbow trout. This modulation could be caused by affecting the viability of the probiotic cell during the process of feed production and intestinal transit or interacting with other bacterial members in the intestine. A high dominant core microbiota at genus level was identified among all the experimental groups. However, the core microbiota was highly modulated regarding relative abundance mainly by diet composition and in a less extent by the probiotic treatment. To further investigate the concept of core microbiota in rainbow trout, Chapter 4 will also investigate the role of antibiotics on microbiota associated with the digesta of rainbow trout and the effect of *P. acidilactici*. Further studies are necessary to investigate specific compound and the diet ingredients responsible for modulation in both *P. acidilactici* and also in intestinal microbiota and what these changes imply for the fish health.

CHAPTER 4. Impact of dietary oxytetracycline treatment and the supplementation of *Pediococcus acidilactici* in the digesta-associated microbiota of rainbow trout

4.1. Abstract

The aims of the current study were to evaluate the effect of oxytetracycline (OTC) and the probiotic bacteria *P. acidilactici* (Bactocell®) on the rainbow trout intestinal microbiota and to assess how a dietary intervention with *P. acidilactici* modulates the effect of OTC on the rainbow trout intestinal microbiota. A 4-week feeding trial was conducted. The experiment involved two phases, the first phase was pre-antibiotic (Pre-Ab) intervention and lasted three weeks, whereas the second phase was during-antibiotic (Dur-Ab) treatment and lasted one week. The groups of fish were kept in a freshwater recirculation system and received one of four different experimental diets; 1) Pre-Ab control diet, without probiotic supplementation; 2) Pre-Ab probiotic diet, supplemented with Bactocell®; 3) Dur-Ab control diet, coated with OTC; 4) Dur-Ab probiotic diet, supplemented with *P. acidilactici* and coated with OTC. Samples of digesta for the microbial characterization using high-throughput sequencing were taken from the distal intestine. The microbiota analysis of the alpha diversity revealed highly significant differences between the Pre-Ab group and Dur-Ab group with lower diversity in the fish group fed the diet containing OTC compared with the group fed the diet without OTC. All experimental groups were dominated by Firmicutes followed by Fusobacteria. This study revealed that OTC substantially modulated the distal intestinal microbiota including impacting multiple members of the core microbiota. However, *P. acidilactici* did not modulate the effect of OTC on the microbiota composition.

4.2. Introduction

Infectious diseases are among the main factors, which hamper the productivity and expansion of aquaculture. The salmonid farming industry is affected by a broad range of bacterial diseases that has caused major economic losses and welfare concerns (Rodger, 2016). For decades, the use of antibiotics in fish and other animals for human consumption have been applied not only to treat bacterial infections but also to improve growth and feed efficiency (Silbergeld et al., 2008; Maron et al., 2013). The use of antibiotics has serious consequences for public health. In 2006 the use of non-medicinal antibiotics supplemented to animals for human consumption was banned in the European Union (EPC, 2003). This decision was mainly led by the finding of a link between the use in the feed of antibiotics in non-therapeutic doses and the increase of prevalence of antibiotic resistance (reviewed by Cogliani et al. 2011). Furthermore, antibiotics are indiscriminate (Cabello, 2006; Burridge et al., 2010), thus after an antibiotic intervention, both pathogenic bacteria and healthy commensal bacteria are eradicated. This lack of specificity can, therefore, impact the microbiota of treated animals. In mammals, frequent use of antibiotics has been associated with intestinal disorders such as diarrhoea and pathogenic infection as reviewed by Keeney et al. (2014). In fish, the use of antibiotics has been associated with disorders affecting the immune system (Romero et al., 2012).

As a result of the constraints related to the use of antibiotics in the animal production industry, including aquaculture, there is a growing interest in alternative methods to prevent infectious diseases and fight against pathogens. One of the methods that have attracted great interest is the use of probiotics. Probiotics have been seen as an alternative to antimicrobials because some

probiotics have been reported to acts as growth promoters (Merrifield et al., 2010b), improve disease resistance (Balcázar et al., 2007) and decrease stress induced by farming conditions (see Section 1.3.3 in Chapter 1). Although there is still a gap of information regarding the mechanisms of action that a probiotic needs to induce in the host to promote their beneficial effects, most researchers accept that probiotic effects are at least partly mediated by improving gut microbiota balance (reviewed by Merrifield and Carnevali 2014) .

A large amount of information evaluating the role of the microbiota in fish and its manipulation to improve health has been published. Different factors have been identified to modulate the microbiota in fish, including diet, environment, seasonality, and dietary supplements such as probiotics (reviewed by Romero et al., 2014). However, relatively few studies have investigated the effect of antibiotics on the gut microbiota of salmonids (Austin and Al-Zahrani, 1988; Moffitt and Mobin, 2006; Bakke-McKellep et al., 2007; Navarrete et al., 2008). These studies are based on the use of culture-dependent techniques, which have limitations to capture the full bacterial diversity in the fish gut. Culture-independent techniques such as high-throughput sequencing (HTS) libraries of the bacterial 16S rRNA gene provide a more comprehensive characterization of the bacterial communities in contrast to culture-dependent techniques (Donachie et al., 2007; Turnbaugh and Gordon, 2008). To the author's knowledge, there are currently no published studies on the impact of antibiotic on gut microbiota using HTS in salmonids.

This Chapter, therefore, aims to i) evaluate the effect of oxytetracycline and *P.s acidilactici* on the rainbow trout intestinal microbiota, and ii) to study how a dietary

intervention with the probiotic bacterial *P. acidilactici* modulate the effect of oxytetracycline on the rainbow trout intestinal microbiota.

4.3. Materials and Methods

4.3.1. Animal husbandry

A 4-week-feeding trial was conducted at the recirculation aquarium facilities at Plymouth University, UK in accordance with the university ethical committee and under the UK Home Office project licence (PPL 30/2644). Prior to initiation of the trial, juvenile rainbow trout (*Oncorhynchus mykiss* Walbaum) were acclimated for one week on a standard commercial diet. At the end of the acclimatisation period, 60 fish (60 ± 1.4 g) were randomly distributed within two fibreglass tanks (150 L capacity) in a density n= 30 fish per tank.

Throughout the experiment, the fish were kept in a freshwater recirculation system (flow rate 100 L/h) with a 12-h dark: 12-h light photoperiod. During the experimental period, the dissolved oxygen level was maintained in 79 ± 2% saturation; the temperature was 16.1 ± 1 °C and the pH 7.1 ± 3. Additionally, ammonia, nitrite and nitrate were measured weekly and maintained at 0.04 ± 0.08 mg/L^{-1}, 0.04 ± 0.02 mg/L^{-1} and 56 ± 2 mg/L^{-1} respectively.

4.3.1. Diet and experimental design

The experiment involved two phases, the first phase was termed as pre-antibiotic (Pre-Ab) and lasted three weeks, whereas the second phase was during-antibiotic (Dur-Ab) and lasted one week. Four experimental diets were designed using the commercial diet (Efico Enviro 930, 3.0 mm pellet BioMar, Denmark) as a basal diet: 1) Pre-Ab control diet, without supplementation; 2) Pre-Ab probiotic diet, supplemented with Bactocell®; 3) Dur-Ab control diet, coated with oxytetracycline (OTC); 4) Dur-Ab control diet, supplemented with Bactocell® and coated with OTC. During the Pre-Ab phase, each tank was assigned to an

experimental diet without OTC i.e. Pre-Ab control diet and Pre-Ab probiotic diet. Whereas during the Dur-Ab phase the fish were fed with OTC diets i.e. Dur-Ab control diet and Dur-Ab probiotic diet. Bactocell® was incorporated into the experimental diet at a dose of 5 x 10^6 CFU/ kg by spraying a 50 ml solution of Bactocell® in phosphate buffer solution into the diet. Antibiotic OTC (Aquanet, Pharmaq, UK) was added to the diet using the oil as a carrier coated on the diets by mechanical mixing for two hours. Oxytetracycline was added at 1% of feed weight to provide a dose of 375 mg kg^{-1} body weight per day. All the fish were fed at a rate of 1.5% body weight per day, based on total tank weight.

4.3.2. Sample collection

In the course of the experiment, samples were obtained at two time-points. The first sampling was in the Pre-Ab phase during feeding the fish with control and probiotic diets for three weeks. The second sampling was during the Dur-Ab phase at week four, i.e. after feeding the fish with Dur-Ab control and Dur-Ab probiotic diet for seven days. A total of 20 fish from each group were sampled (five fish from each tank at each sampling point, n = 5). The intestines were aseptically removed using sterile instruments and divided into the proximal and distal intestine. The distal intestinal digesta from individual fish were collected into sterile tubes. The dissection and sampling methodologies are described in section 2.3

4.3.3. Microbiological analysis

For analysis of microbiota, samples of digesta from five fish per tank (n = 5) were used. The sampling was conducted as described in Section 2.3. and the analysis was conducted according to Section 2.4.

4.3.4. Statistical analysis

Statistical comparisons in all the analyses were conducted by two way ANOVA for alpha diversity and two way PERMANOVA for beta diversity. To evaluate the effect of probiotic on specific bacterial taxa, only control and probiotic groups in the Pre-Ab phase were used in the LEfSe analysis. Meanwhile, all the experimental groups were taken into account in the LEfSe analysis to evaluate the effect of the antibiotic factor on specific taxa. Statistical analysis was carried out using the methods described in section 2.5.

4.4. Results

4.4.1. High-throughput sequencing data

Twenty samples of digesta from distal intestine were processed on the Ion Torrent platform to investigate the bacterial microbiota associated to the digesta. One sample from Dur-Ab control group was discarded due to difficulties in the PCR amplification. High-throughput sequencing generated 771,280 reads before quality control (40,593 ± 6,363 reads per sample). After quality filtering, processing the data in QIIME, filtering spurious sequences and discarding reads affiliated to Streptophyta a total of 725,842 reads were retained (38,202 ± 5,937 reads per sample). Removed reads belonging to Streptophyta were relatively low (1.24 ± 0.64%) except for one sample from the sample number 17 from Dur-Ab control group which Streptophyta reads accounted for 52.3%.

4.4.2. Effect of probiotic and oxytetracycline on the distal intestinal microbiota

The alpha diversity parameters Chao1 and Phylogenetic diversity (PD) revealed highly significant differences between the Pre-Ab group and Dur-Ab group (P = 0.01) (Figure 4.1.). Shannon index also showed significant statistical differences between the experimental Pre-Ab and Dur-Ab groups (P = 0.014). The fish group fed the OTC diet displayed lower alpha diversity in all the alpha diversity parameters evaluated compared with the group fed the diet without OTC. No significant differences in alpha diversity were observed between the control and probiotic groups regardless of the use of OTC in the diet. No interaction between the factors studied, i.e. probiotic and antibiotic factors (Chao1 P = 0.584, PD P = 0.276) were detected after Analysis of variance (ANOVA).

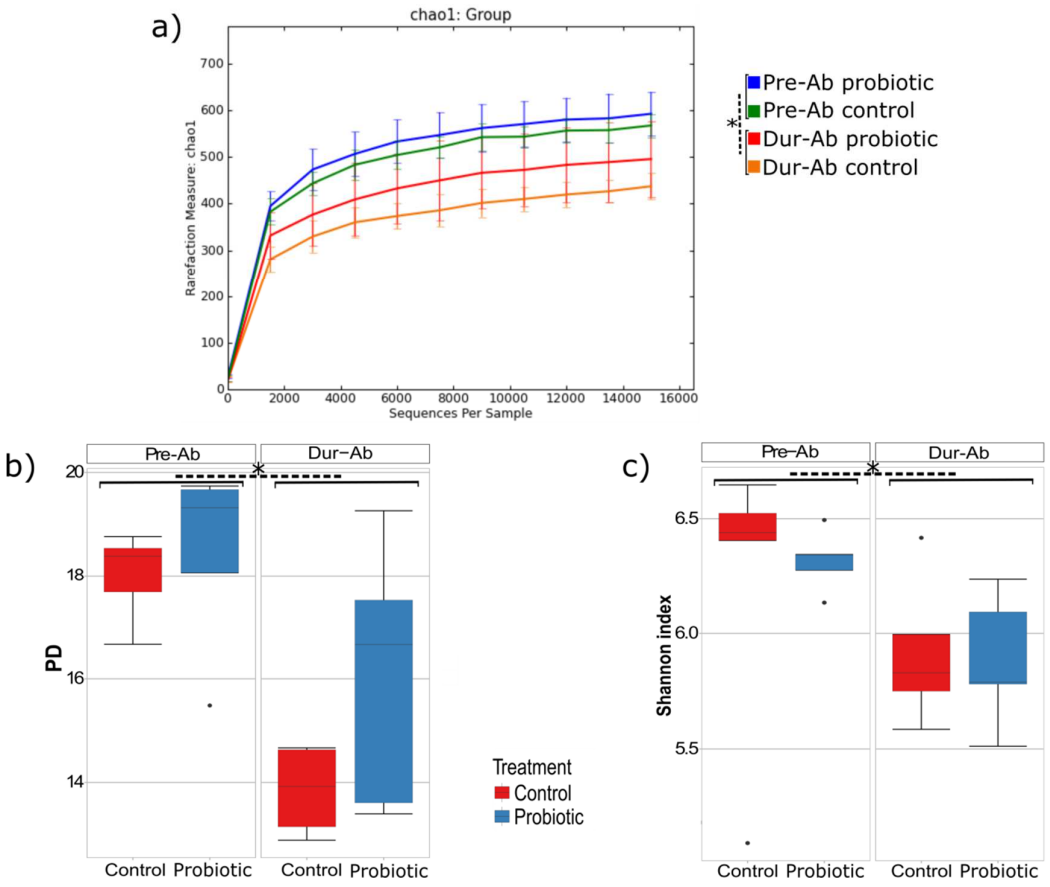


Figure 4.1. Alpha diversity parameters of the distal intestinal microbiota associated to digesta comparing pre-antibiotic (Pre-Ab) and during antibiotic (Dur-Ab) groups. a) Rarefaction curve based on Chao1 metric representing the average and standard deviation (error bars) of OTUs per experimental group; b) Phylogenetic diversity (PD) boxplot; c) Shannon index boxplot. Statistical analysis was conducted using alpha diversity parameters from samples rarefied at an even depth of 15,000 sequences. Statistical differences are denoted by asterisk * ($P < 0.05$). Dashed lines represent significant differences between Pre-Ab and Dur-AB groups. No significant differences were observed between control (red) and probiotic (blue).

Beta diversity based on weighted and unweighted UniFrac dissimilarity matrix was used to compare the effects of probiotic *P. acidilactici* and OTC on the overall bacteria composition in the distal intestine, and the results are visualised in Figure 4.2 by a principal coordinate analysis (PCoA) and statistically analysed by PERMANOVA (Table 4.1.). The principal coordinate analysis from weighted and unweighted UniFrac showed that the samples clustered by OTC factor. Two clusters are evident, the first cluster is composed of the samples from the Pre-Ab

groups, whilst the second cluster is composed of the samples from Dur-Ab groups. No clear clustering effect was observed between control and probiotic groups. PERMANOVA analysis is consistent with the PCoA results, revealing significant differences between the Pre-Ab and Dur-Ab experimental groups but not between control and probiotic groups. Higher variations between samples were detected within Dur-Ab group than within Pre-Ab groups as shown by PCoA.

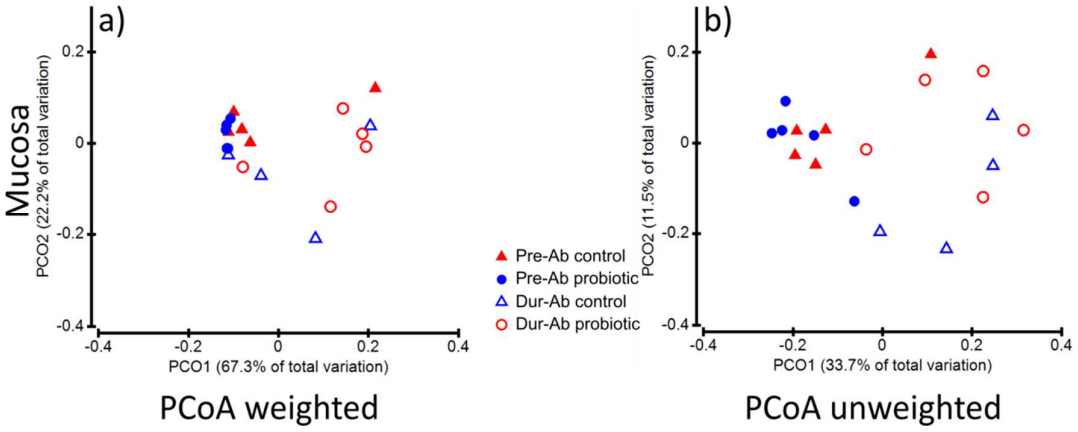


Figure 4.2. Principal coordinate analysis (PCoA) of the distal intestinal microbiota associated to digesta using UniFrac distances. The percentage of variation is explained by PC1 and PC2 axis. Each plot represents the differences among the different experimental groups. Pre-antibiotic control (Pre-Ab control, red triangles), pre-antibiotic probiotic (Pre-Ab probiotic, blue circles), During-antibiotic control (Dur-Ab control, blue triangles) and During-antibiotic probiotic (Dur-Ab probiotic, red circles). a) PCoA weighted UniFrac; b) PCoA unweighted UniFrac.

Table 4.1. PERMANOVA results from weighted and unweighted UniFrac.

Group comparison	**PERMANOVA**			
	Weighted UniFrac		Unweighted UniFrac	
	Pseudo-F/t	*P*	Pseudo-F/t	*P*
Probiotic vs Control	0.29	0.848	0.84	0.704
Pre-Ab vs. Dur-Ab	6.61	0.002	5.39	0.002
Interaction	2.19	0.125	1.17	0.253

The relative abundance at phylum and class levels is displayed in Figure 4.3. Five phyla accounted for 95% of the total reads for all the samples evaluated with important changes according to the experimental group. The most dominant group was the phylum Firmicutes, specifically the class Bacilli which accounted for 63 ± 7.5% of the total abundance in all the experimental groups. The phylum Fusobacteria was the second most abundant taxon accounting for 11.8 ± 12.2% of the total sequences of all the experimental groups. The genus *Pediococcus* had a significantly higher abundance in the Pre-Ab probiotic group (2.65 ± 1.1%) than in the Dur-Ab probiotic group (0.57 ± 0.6).

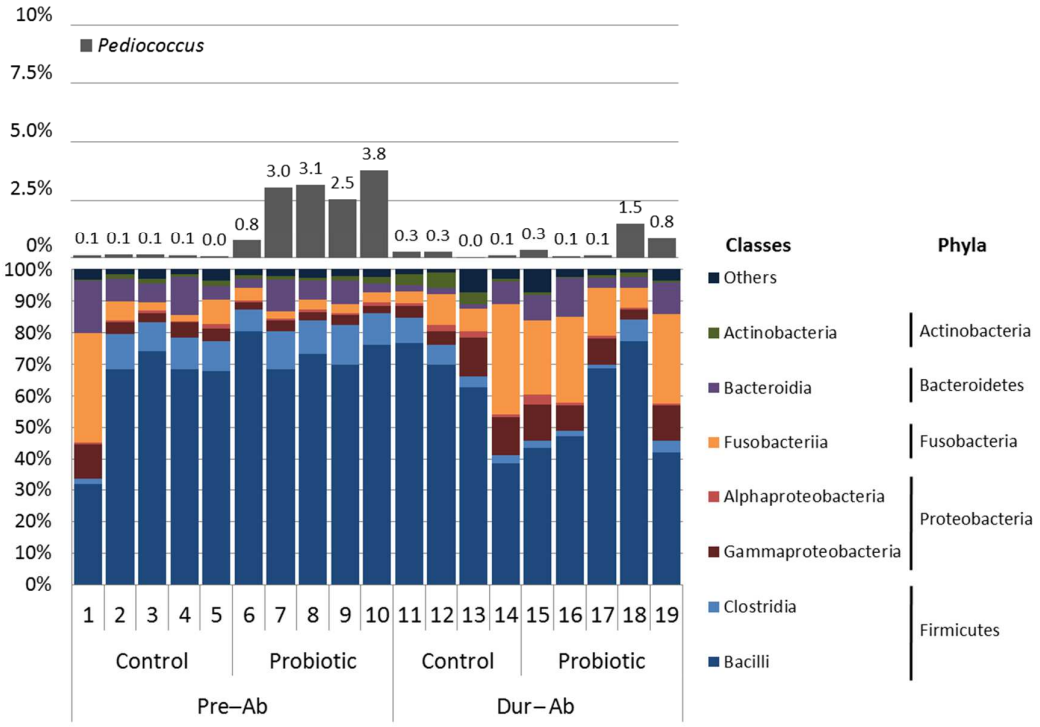


Figure 4.3. Relative abundance of the main bacterial taxa at class and phylum level and abundance of the genus *Pediococcus*. Classes below an abundance average of 0.5% per experimental group are not shown but summarised in a mixed group "Others". Numbers below the bars represent the number of the fish for each experimental group. Pre-antibiotic (Pre-Ab), during-antibiotic (Dur-Ab).

LEfSe was conducted to detect the significant differences in specific taxa modulated by the factors investigated in this study i.e. probiotic and antibiotic factors. The differences in taxa between Pre-Ab and Dur-Ab were assessed in

LEfSe using all the samples, and the results are displayed in Figure 4.4. Meanwhile, the effect of probiotic on specific taxa was only evaluated in samples from the Pre-Ab phase. The comparison between control and probiotic in samples from Dur-Ab phase was avoided since, according to PCoA, no clustering effect was observed in this phase.

A high number of taxa was modulated in the bacterial microbiota associated to digesta by the addition of OTC in the diet (44 genera enriched in the Pre-Ab group and 14 genera enriched in the Dur-Ab group). The main changes associated to the OTC in the Dur-Ab groups were an enrichment of the phyla Proteobacteria and Fusobacteria and a decreased abundance of Firmicutes. A significant enrichment of the phylum Fusobacteria was led mainly by overrepresentation of the genus *Cetobacterium*. In the phylum Proteobacteria, the main class, significantly enriched in the Dur-Ab groups was Gammaproteobacteria (genera *Edwarsiella* and *Pseudomonas,*). Although, most of the taxa belonging to the phylum Firmicutes decreased in the Dur-Ab groups a few exceptions were observed with the genera *Lactobacillus*, *Enterococcus* and *Bacillus,* which were enriched during the antibiotic intervention with OTC. The reduction in the relative abundance of different taxa from the phylum Firmicutes in the Pre-Ab group in contrast with the Dur-Ab group involved different taxonomic groups from the class Clostridia (genera *Helcococcus*, *Peptoniphilus*, *Clostridium, Hespellia*). Furthermore, other taxa from phylum Firmicutes belonging to the LAB group were also significantly decreased in the Pre-Ab groups, the main genera affected were *Lactococcus*, *Vagococcus*, *Weissella* and *Carnobacterium*. In contrast to the OTC intervention, the supplementation of the probiotic *P. acidilactici* had a mild modulatory effect on specific taxa, as only 10 genera were significantly different between control and probiotic groups during the Pre-Ab phase. The main phyla

modulated in the probiotic group were Proteobacteria and Chloroflexi, which had lower abundance in the probiotic group, whereas the phylum Firmicutes was significantly enriched. Regarding the phylum Proteobacteria, *Enterovibrio*, and two unidentified OTUs from the families *Enterobacteriaceae* and *Aeromonadaceae* were the main taxa reduced in the probiotic group, whilst the reduction in the phylum Chloroflexi in the probiotic group was led by a decline of the genus *Ardenscatena*. The main Firmicutes taxa significantly enriched in the probiotic group belonged to the order Lactobacilliales (genera *Streptococcus* and *Pediococcus*) and Bacilliales (genera *Virgibacillus* and *Brochothrix*).

To evaluate the core microbiota, the shared OTUs in 80% of the samples at the genus level were determined for each of the four experimental groups (Figure 4.6.). The core microbiota was represented by 33 OTUs which accounted for the 20.6% of all the shared OTUs among all the experimental groups. Taking together all the OTUs of the core microbiota, they accounted for a large proportion of the total abundance of all the experimental groups i.e. >82%. The core microbiota was represented by four phyla Bacteroidetes, Firmicutes, Fusobacteria and Proteobacteria. The phylum Firmicutes was the dominant phylum in the core microbiota regarding the number of OTUs (18 OTUs) and the relative abundance (>43% in all the groups), followed by Proteobacteria. Members of the core microbiota were largely modulated by the factors investigated in this study mainly by OTC intervention as revealed by LEfSe.

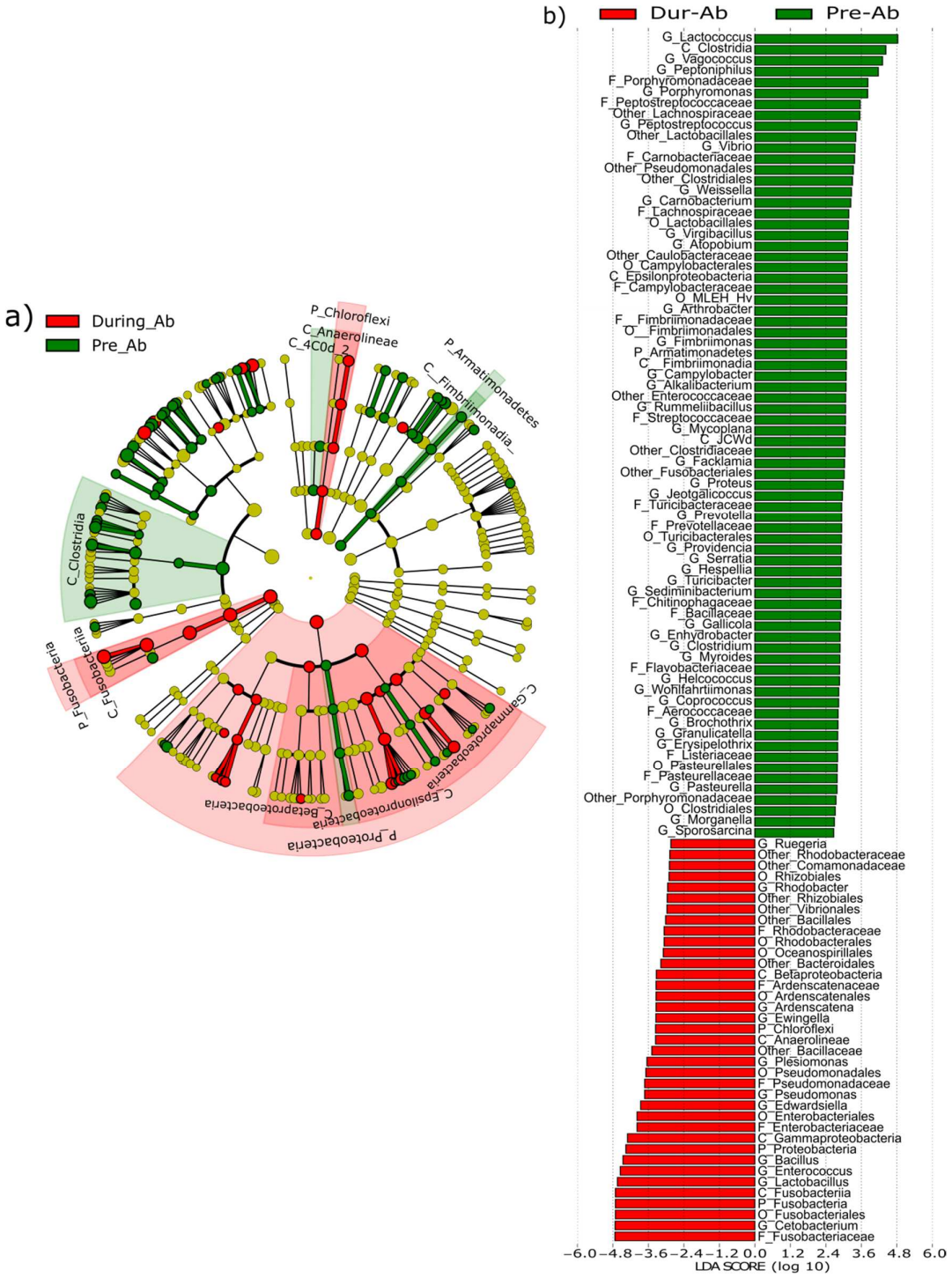


Figure 4.4. Taxonomic differences in distal intestinal microbiota from digesta between control and probiotic groups according to LEfSe analysis. The analysis was carried out with the relative abundance of all samples at the genus level. The pre-antibiotic (Pre-Ab) and during antibiotic (Dur-Ab) groups were treated as classes and probiotic and control groups as subclasses. a) A circular cladogram is representing the significant enriched OTUs between Dur-Ab (red) or Pre-Ab (green) groups. No significantly different OTUs are represented in yellow. The diameter of each dots is proportional to its effect size. b) Linear discriminant analysis (LDA), differentially enriched OTUs are arranged in descending order according to LDA score.

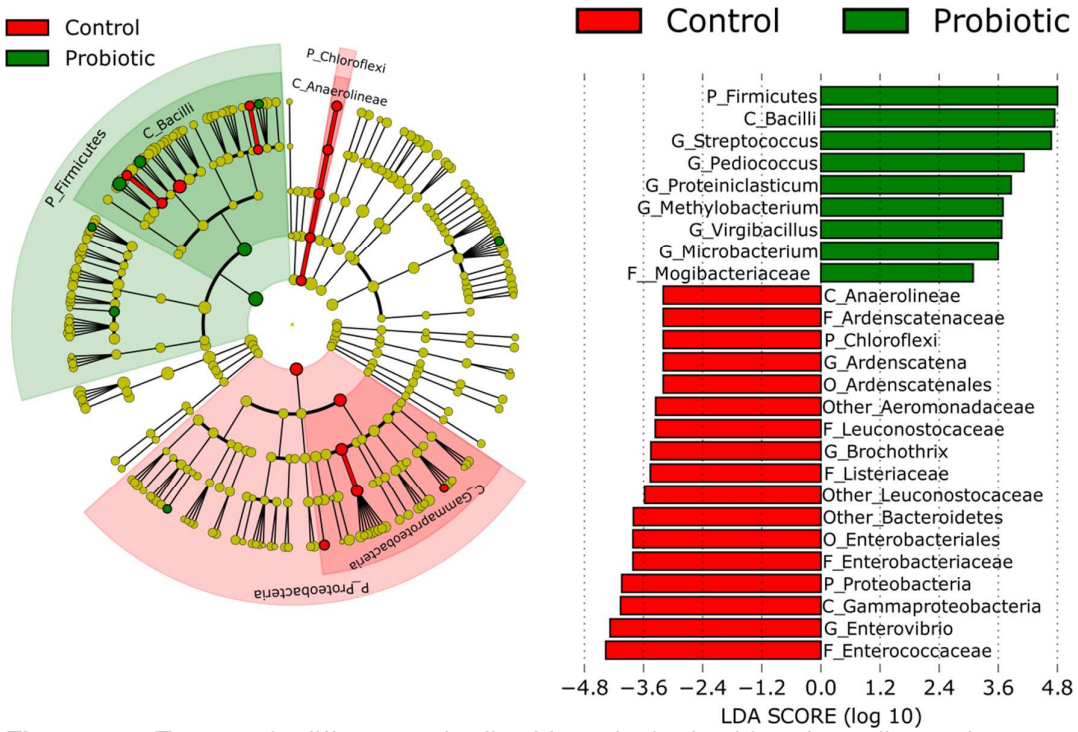
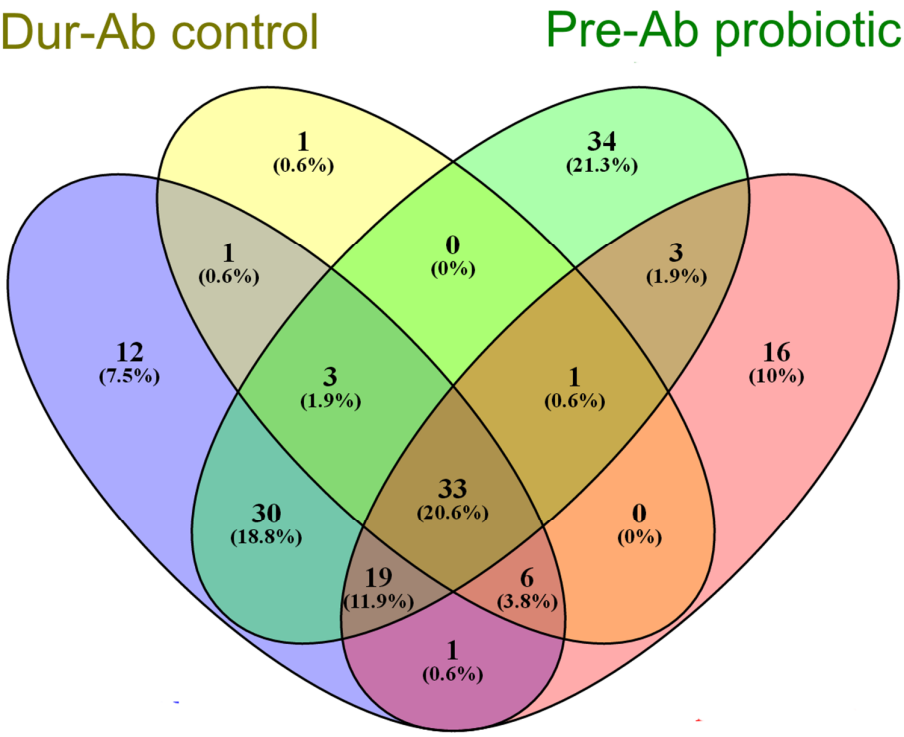


Figure 4.5. Taxonomic differences in distal intestinal microbiota from digesta between control and probiotic groups according to LEfSe analysis. The analysis was carried out with the relative abundance of the samples from the Pre-antibiotic group at the genus level. Probiotic and control groups were treated as classes. a) A circular cladogram is representing the significant enriched OTUs between control (red) or probiotics (green) groups. No significantly different OTUs are represented in yellow. The diameter of each dots is proportional to its effect size. b) Linear discriminant analysis (LDA), differentially enriched OTUs are arranged in descending order according to LDA score.

a)



b)

Core Taxonomy		Number of OTUs in the order	Average abundance (%)			
			Pre-Ab		Dur-Ab	
Phylum	Order		Control	Probiotic	Control	Probiotic
Bacteroidetes	Bacteroidales	2	7.34	4.32	3.12	7.22
Firmicutes	Unidentified Firmicutes	1	0.24	0.10	0.24	0.26
	Bacilliales	3	1.87	1.35	6.88	8.33
	Lactobacilliales	12	56.23	65.80	51.04	43.64
	Clostridiales	3	4.81	6.10	2.39	1.39
Fusobacteria	Fusobacteriales	2	10.03	2.62	13.42	19.82
Proteobacteria	Aeromonodales	2	0.69	0.44	0.63	1.00
	Alteromonadales	1	0.51	0.39	2.10	0.32
	Enterobacteriales	4	2.05	0.93	2.87	4.43
	Pseudomonadales	1	0.81	0.31	1.37	1.80
	Vibrionales	2	0.12	0.09	0.30	0.24
	Total	33	84.71	82.44	84.36	88.45

Figure 4.6. Core microbiota of distal intestinal digesta. a) Venn diagram showing the shared OTUs across 80% of the samples per experimental group, including control and probiotic in two phases, pre-antibiotic (Pre-Ab) and during antibiotic (Dur-Ab). b) Table showing the contribution of each component (average abundance) of the core microbiota in each experimental group.

4.5. Discussion

Antibiotics have been widely used in aquaculture as a tool to counteract the effect of bacterial infections (Cabello, 2006; Burridge et al., 2010). The addition of antibiotics in the diet is well recognized as a major disturbing factor of the gut microbiota in humans, (Zeissig and Blumberg, 2014), poultry (Lin et al., 2013; Videnska et al., 2013) and swine production (Looft et al., 2014; Schokker et al., 2015); however, in salmonid aquaculture the impact of antibiotics on the intestinal microbiota is poorly understood. In this study, the influence of a dietary supplementation of a commercial relevant antibiotic, OTC, on the intestinal microbiota of rainbow trout was evaluated. Furthermore, the potential role of a probiotic supplementation to ameliorate the alteration in the intestinal microbiota by OTC was investigated. The results suggested that the OTC had a larger impact on the intestinal microbiota than the probiotic *P. acidilactici* and the use of a probiotic did not modulate the effect of OTC on the bacteria communities of the distal intestine.

4.5.1. Effect of oxytetracycline and probiotic supplementation on the bacterial microbiota of the distal intestine

Some studies in fish have demonstrated that antibiotics have a significant impact on the intestinal microbiota of salmonids (Moffitt and Mobin, 2006; Bakke-McKellep et al., 2007; Navarrete et al., 2008). In this study, the addition of OTC in the diet significantly reduced the alpha diversity indices of the digesta-associated bacterial communities. This result is in line with the research conducted by Bakke-McKellep et al. (2007) and Navarrete et al. (2008) in Atlantic salmon. These authors reported that OTC reduced the diversity of cultivable

bacteria in the intestine. The antibiotic OTC belongs to the tetracycline group, which are broad-spectrum antibiotics with effects on different bacterial species. Thus, the significant decrease in alpha diversity in the fish group fed OTC compared with the untreated group is not surprising. On the other hand, the probiotic *P. acidilactici* did not affect the alpha diversity of the Pre-Ab nor Dur-Ab groups. This finding are similar to the results reported in Chapter 3, which indicated that *P. acidilactici* did not reduced the alpha diversity of the microbiota associated to digesta in rainbow trout fed FM and PMIX diets.

The impact of OTC and probiotic supplementation on the beta diversity of the trout gut microbiota was evaluated by UniFrac; the findings were consistent with the results reported in alpha diversity. Thus, the greatest impact on the bacterial communities was induced by OTC. Meanwhile, the factor probiotic only had a subtle effect on the distal intestinal microbiota. Similar results between the weighted and unweighted UniFrac suggested that OTC had an impact not only on the relative abundance of the bacterial microbiota before and during OTC administration but in the presence or absence of specific OTUs. The experimental groups Pre-Ab and Dur-Ab when evaluated by PCoA revealed that these two groups differed not only in different clustering patterns but also in a larger inter-individual variation within Dur-Ab group compared with the Pre-Ab group. This finding suggested that the microbiota disturbances associated with OTC administration were individual dependent and the final bacterial composition could be the result of multiple factors such as differences in the initial bacterial composition, different individual exposure to OTC and complex bacterial interactions in the intestine after OTC exposition.

Although the overall differences between control and probiotic groups were not significant, the PCoA analysis showed a mild clustering effect in the Pre-Ab group. Furthermore, the supplementation of *P. acidilactici* did not ameliorate the significant disturbing effect of OTC in the bacterial communities associated to the distal intestinal digesta. A strong reduction of the relative abundance of *Pediococcus* in the fish fed the probiotic diet during the antibiotic phase (Dur-Ab) was observed in the probiotic group compared to the probiotic group before OTC administration. This finding could explain the lack of modulation between probiotic and control group in the Dur-Ab phase, despite that this experimental group was fed the probiotic diet uninterruptedly during this phase. As reported in other studies the potential effect of a bacterial probiotic in the host could be mediated by several factors such as diet (Chapter 3)(Tachon et al., 2014), probiotic doses (Ramos et al., 2015) among others. The lower level of *Pediococcus* in fish fed the OTC diet could be the result of the direct effect of the antibiotic on the survival of the probiotic *P. acidilactici.* Although some strains of *P. acidilactici* are intrinsically resistant to tetracycline, generally this species is susceptible to OTC (Temmerman et al., 2003; Barbosa et al., 2015).

4.5.2. Core microbiota is modulated by OTC

Prior to the antibiotic administration, the phylum Firmicutes was the dominant taxon in the distal intestinal digesta of rainbow trout. This is in agreement with the results found in Chapter 3, despite the fact that the ingredients composition varied widely between both studies (i.e. commercial diet in this chapter vs. FM, SB and PMIX diets in chapter 3). The dominance of Firmicutes in the microbiota associated to the digesta in both studies (Chapter 3 and Chapter 4) suggests that members of this phylum are well adpated to distal intestine of rainbow trout. In

this study, a core microbiota of 33 OTUs at genus level was identified across different experimental groups. The class Bacilli from the phylum Firmicutes was the main taxon regarding the relative abundance and number of OTUs represented in the core microbiota. The high abundance of members of Bacilli class as part of the core microbiota associated to the digesta found in this study as well as previous results from Chapter 3 indicating the dominance of Bacilli class in the core microbiota associated to digesta suggest that this taxon has a central role in the distal intestine of rainbow trout. Lyons et al. (2017) reported that the class Bacilli was the major taxon present in the mucosa of rainbow trout under farming conditions. However, the relative abundance of the class Bacilli reported in the study above was much lower (16.8%) than that reported in this study (63%). Members of the phyla Fusobacteria, Proteobacteria and Bacteroidetes, were also identified as part of the core microbiota, which is in agreement with previous studies in rainbow trout (Wong et al., 2013; Etyemez and Balcázar, 2015; Lyons et al., 2017). The identification of a core microbiota is of interest in the study of the gut microbiota of animals because these are well adapted bacterial members that are shared by a significant number of healthy individuals and could have a more important role in hosting health in comparison with transient microbes (Salonen et al., 2012; Shade and Handelsman, 2012). Despite that a core microbiota was identified in all the experimental groups in this study, the relative abundance of some of these shared OTUs was significantly different among experimental groups. The fish group fed OTC showed the greatest changes in OTUs belonging to the core microbiota, whereas the fish group fed probiotic diet had only a few core microbiota taxa modulated. Overall, the antibiotic factor was the stronger driver of the distal intestinal microbiota associated to the digesta, causing perturbation in a large number of important

bacterial taxa. The mild effect of the probiotic factor in the microbiota between the Pre-Ab control group and Pre-Ab probiotic in this study compared with the results observed in Chapter 3 in digesta microbiota could be explained by the potential role that different diet ingredients used in both studies may have in promoting or inhibiting the metabolism and survival of *P. acidilactici* in the intestine.

4.5.3. Conclusion

Alterations in intestinal microbiota induced by antibiotics have been associated with several disorders in mammals. Even though the intestinal health of rainbow trout was not evaluated in this study, disruption of the intestinal microbiota balance including members of the core microbiota induced by OTC administration suggests that this antibiotic could potentially predispose the host to different intestinal disorders. Other probiotics with resistance to OTC should be evaluated in the future to evaluate their potential to ameliorate the adverse effect of this antibiotic on intestinal microbiota. Future studies are also necessary to examine the dynamic and extent of the intestinal microbiota subsequent to the exposure to OTC and other antibiotics in rainbow trout and the predisposition to the development of antibiotic-associated diseases. The current Chapter together with Chapter 3 have investigated the influence of *P. acidilactici* in the intestinal microbiota of rainbow trout which is a good model to study probiotics in freshwater. In order to study the role of *P. acidilactici* in seawater, the two following Chapters will use Atlantic salmon as a model and focus also on studying the microbiota associated to the intestinal mucosa.

CHAPTER 5. Microbiota and intestinal health of Atlantic salmon during smoltification and the influence of dietary supplementation of *Pediococcus acidilactici*

5.1. Abstract

The aim of this study was to assess the effect of the transfer from freshwater to seawater on the bacterial communities of the distal intestine of Atlantic salmon and also to evaluate the microbiota modulation in these fish when the bacteria *P. acidilactici* MA18/5M was added to the diet. Additionally, the effect of probiotic supplementation on the histology and the expression of selected immune genes was also investigated. A 12-week feeding trial was conducted in a flow-through rearing system involving 5 weeks in freshwater and 5 weeks in seawater. Fish received one of two diets: one control diet and one probiotic diet. Samples from the digesta and mucosa were taken during freshwater and seawater stages for bacterial characterization. Intestinal health was evaluated by histology and gene expression. The main phyla detected in the distal intestine of Atlantic salmon during both freshwater and seawater stages were Firmicutes, Proteobacteria, Fusobacteria and Actinobacteria. Significant differences were observed in the intestinal microbiota between the digesta and mucosa. Results from gene expression analysis revealed an up-regulation of *tlr3*, *tnf-α* and *mx-1* in the distal and proximal intestine of salmon in seawater stage. Dietary probiotic supplementation and transfer from freshwater to seawater had a substantial impact on the microbial communities of the distal intestine of Atlantic salmon. However, the extent of the microbiota modulation was greater in the mucosa than in digesta. Moreover, the changes observed in fish fed the *P. acidilactici* diet after seawater transfer in the bacterial microbiota associated to the mucosa were associated with a potential activation of the antiviral response.

5.2. Introduction

Parr-smolt smoltification is a crucial stage in the life cycle of salmonids during the transition from freshwater (FW) to seawater (SW) (Björnsson et al., 2012). Smoltification in Atlantic salmon involves complex physiological, morphological, endocrine and neural changes in the smolts to be adapted for life in seawater (Sundh et al., 2014). The central role that the intestine plays in the adaptation of fish to the new seawater environment, especially regarding the osmoregulation process, is widely recognised (Hoar, 1988; Veillette et al., 2005; Jutfelt et al., 2007). In addition to the essential functions of nutrient digestion and absorption and acting as a physical and immunological barrier, during the smoltification process, the intestine is involved in maintaining the osmotic homoeostasis desalinating absorbed seawater in order to avoid dehydration (Grosell, 2010; Whittamore, 2012).

Chronic and acute stressors have been recognised to negatively affect the intestinal barrier function of fish (Olsen et al., 2002; Sundh et al., 2010; Niklasson et al., 2011). The transfer of fish from a freshwater to a new marine environment is recognised to be a stressful period in the life cycle of Atlantic salmon. As a consequence, fish are more susceptible to be affected by pathogens and handling stress (Roberts and Pearson, 2005). The entry of smolts to the new marine environment involves the confrontation with several pathogens that are mainly prevalent in seawater such as infectious pancreatic necrosis virus (IPNV), infectious hematopoietic necrosis virus (IHNV) infectious salmon anaemia virus (ISAV) and pancreas disease virus (PDV) (Lafferty et al., 2015; Rodger, 2016).

The intestine of fish harbours a broad consortium of different microorganisms (bacteria, viruses, yeasts, archaea and protozoans) that have an active

interaction with the intestine. Previous studies have focused on the bacterial microbiota, describing the importance that these organisms have on the host, including the production of enzymes, growth performance, immunity and disease resistance (reviewed by Romero at al., 2014). Despite the advances in understanding the microbial ecology in the gut of salmonids, little information is known about the changes in the microbial communities during the adaptation of Atlantic salmon to seawater environment or the potential effect that probiotic may have during this critical phase.

P. acidilactici MA18/5M is used as a probiotic for fish in freshwater and seawater environments. It has been reported that the use of *P. acidilactici* MA18/5M as a dietary supplement has led to improvements in the gut health of rainbow trout (Merrifield et al., 2010d) and Atlantic salmon (Abid et al., 2013; Vasanth et al., 2015). Previous research has also demonstrated that dietary supplementation of the probiotic *P. acidilactici* MA18/5M can modulate the intestinal microbiota (Chapter 3) of fish and stimulate various non-specific immunological parameters (Ferguson et al., 2010; Standen et al., 2013).

Therefore, the aim of this study was to assess the effect of the transfer from freshwater to seawater on the bacterial communities of the distal intestine of Atlantic salmon and also to evaluate the microbiota modulation in these fish when the probiotic *P. acidilactici* MA18/5M was added to the diet. Additionally, the effect of probiotic supplementation on the histology and the expression of selected immune genes was investigated.

5.3. Materials and methods

5.3.1. Animal husbandry

The trial was conducted at the Aquaculture Research Station Kårvika, Tromsø (Norway) using a flow-through rearing system. A fish batch of 900 Atlantic salmon parr was randomly allocated into six 500 L tanks (150 fish per tank, average weight 30 g). Five weeks before the start of the experiment, fish were fed a commercial diet and acclimated to a 24 hours light regime and 12 °C temperature. The feeding trial lasted 10 weeks involving 5 weeks in freshwater and 5 weeks after smoltification in seawater. During the trial, the oxygen saturation was kept over 85% throughout the experiment.

5.3.2. Diets and experimental design

Two iso-nitrogenous and iso-lipidic diets were formulated according to the nutritional requirements of Atlantic salmon. The composition of the two experimental diets (one control diet and one probiotic diet) used in this trial is displayed in Table 5.1. Both diets were made by BioMar AD (Denmark). The trial was run in triplicate, randomly allotting the tanks for each experimental group. The probiotic group was fed a diet supplemented with Bactocell® (*P. acidilactici*), and the control group received the same diet without supplementation. The recovery of *P. acidilactici* in the probiotic diets was 1.19 x 10^6 CFU/g. Experimental groups were fed from Monday to Sunday at satiation using an automatic feeder. To achieve this, fish were fed for 4 hours per day in excess of 10-15% of expected feed consumption per day.

Table 5.1. Composition for control and probiotic diets during freshwater and seawater stages.

	Freshwater		Seawater	
Ingredients (%)	**Control**	**Probiotic**	**Control**	**Probiotic**
Marine protein	46.7	46.9	40.0	40.
Vegetable protein	24.53	23.95	28.5	28.5
Binder	11.5	11.5	12.0	12.0
Vegetable oil	11.75	11.75	5.0	5.0
Fish oil	5.04	5.04	11.8	11.8
Vitamin and mineral mix	2.01	2.01	3.15	3.15
Bactocell®	0	0.03	0	0.03
Chemical composition (%)				
Crude protein	48	48	22	22
Crude lipid	22	22	45	45
Pellet size (mm)	2.8	2.8	3.5	3.5

All dietary ingredients were sourced from BioMar's routine suppliers (not listed here for commercial reasons.

5.3.3. Sample collection

During the experiment, samples were obtained in two time-points. The first sampling was during the freshwater stage one week before the transfer to seawater. The second sampling was during the seawater stage at week 10, i.e., 5 weeks after transfer to seawater. A total of 18 fish from each group (six from each tank) were sampled at each sampling point. Fish dissection and sampling methodology are described in Section 2.3.

5.3.4. Microbiological analyses

For analysis of the distal intestinal microbiota, 6 fish were sampled as described in Section 2.3. and analysed according to Section 2.4. In each sampling point, digesta samples were pooled per tank (3 samples per treatment), whereas mucosa samples were taken from individual fish (6 samples per treatment).

5.3.5. Gene expression analysis

Distal intestine from 5 fish, i.e. 2 from two tanks and 1 from the third tank and sampling point, were sampled for gene expression analysis. Each target gene

was normalised using the geometric average expression of two reference genes (elongation factor 1 and beta-actin). RNA extraction, cDNA synthesis, real-time PCR and data analysis were carried out as described in Section 2.5. The primer sequences of the genes evaluated in this study are presented in Table 5.2.

Table 5.2. List of primers used for the gene expression in the present study.

Gene	Primer Sequence (5'-3')	Annealing temperature (°C)	Primer efficiency	Amplicon Size (bp)	GeneBank number
ef-1a	F-TCTTGGTCGTTTTGCTGTGC R-AGCCTTGATGACACCGACAG	60	1.8	61	AF321836
actin	F-TCAGGGAGTGATGGTTGGGA R-GCCACTCTCAGCTCGTTGTA	60	2.1	171	NM_001123525.1
tnf–a	F-ACACACTGGGCTCTTCTTCG R-GCACTTGACCCTAAACGAAGC	58	2.1	52	NM_001123589.1
pcna	F-ACAGTTGTGTGGTCAGGATGC R-GAACTTAACGCCATCCTTGG	60	1.9	110	BT056931
hsp-70	F-TGGTCCTGGTGAAGATGAGG R-TGGCCTGTCTCTGTGAATCG	60	1.9	108	AJ632154
tlr-3	F-CTCTAACGGCAACCAGAAGC R-ATGGTGAGGTTGGACAGAGG	60	2.0	144	BK008646
mx-1	F-AAGCTGGCAGAGACACATGC R-ACATCCTTTCTGCCGAGTCC	60	1.9	73	NM_001123693

5.3.6. Intestinal histology

Distal intestinal tissue from 3 fish per tank and sampling point was processed as described in Section 2.8.1. Images from histology were taken from each intestine section and analysed with the software Image J version 1.36 (National Institutes of Health, USA). The average of mucosa fold length was measured in at least 15 well-oriented folds per section stained with haematoxylin and eosin. Fold length was only measured in primary folds with at a minimal length of 200 µm, complex folds were not taken into account. Goblet cells were counted in alcian blue – PAS stained sections and counted across a distance of 200 µm in at least 5 folds per section and averaged. The perimeter ratio (PR) of each intestinal section (arbitrary units, AU) was measured using the external perimeter (EP) and lumen perimeter (LP) and calculated by the formula: PR=LP / EP.

5.3.7. Statistical analysis

Statistical comparisons in all the analyses were conducted between the control and probiotic groups at the same sampling point, except in the microbiological analysis where the effect of the environment was evaluated comparing control groups between freshwater and seawater. Statistical analysis was carried out using the methods described in Section 2.5.

5.4. Results

5.4.1. Microbiota analysis

5.4.1.1. High-throughput sequencing data

An Ion Torrent sequencing platform was used to sequence the variable region 1 and 2 of the 16S rRNA gene. A total of 5.0 million reads was generated from 36 samples sequenced before quality control. After quality filtering, processing the data in QIIME, filtering spurious sequences and discarding reads affiliated to Streptophyta, a total of 1,911,911 reads (53,108 ± 33,097 reads per sample) were retained. The percentage of removed reads belonging to Streptophyta in samples of digesta and mucosa ranged from 0.3 to 2.2% except for the digesta samples from seawater stage, which had a significantly higher percentage of removed reads (i.e. 41.8 and 44.7% for probiotic and control groups, respectively).

5.4.1.2. Intestinal microbiota of distal intestine

To assess whether the composition of the bacterial communities in the distal intestine was influenced by the supplementation of the probiotic in the diet, several comparisons were performed using alpha and beta diversity metrics. Since the digesta samples were pooled per tank and mucosa samples were taken per individual fish, the comparisons were focused on evaluating the differences between control and probiotic groups during the freshwater and seawater stages. However, some analyses were conducted to highlight major differences in the bacterial composition between digesta and mucosa.

Alpha diversity parameters were evaluated using Chao 1, PD and Shannon parameters (Figure 5.1.). The rarefaction curve based on the Chao 1 index reached the plateau, suggesting that the sequencing depth had a sufficient

coverage to evaluate the diversity of both digesta and mucosa samples. In overall, there was a trend toward decreased diversity during seawater stage in comparison with freshwater in both digesta and mucosa samples. All alpha diversity parameters showed that the diversity in the mucosa was significantly higher in the control group compared with the probiotic group in seawater. The Shannon index also revealed that the probiotic group had a significantly higher diversity of bacterial communities associated to the digesta than the control group.

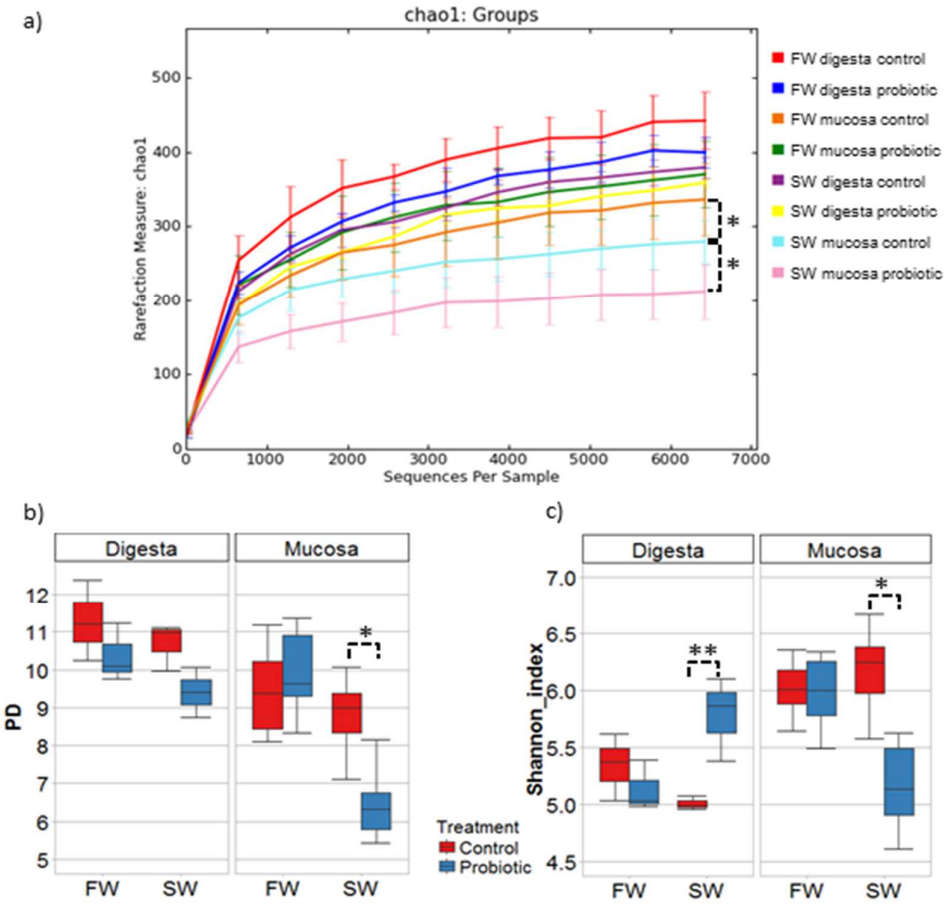


Figure 5.1. Alpha diversity parameters of the distal intestinal microbiota comparing probiotic and control groups in freshwater (FW) and seawater (SW) stages and mucosa and digesta. a) Rarefaction curve based on Chao1 metric representing the average and standard deviation (error bars) of OTUs per experimental group; b) Phylogenetic diversity (PD) boxplot; c) Shannon index boxplot. Statistical analysis was only conducted to compare differences between control and probiotic group using alpha diversity parameters from samples rarefied at an even depth of 6,435 sequences. Statistical differences between control and probiotic group are denoted by asterisks * ($P < 0.05$) and ** ($P < 0.01$).

Comparisons between experimental groups using weighted and unweighted UniFrac revealed substantial differences in the bacterial composition as shown by PCoA plots and PERMANOVA analysis of the mucosa and digesta-associated microbiota (Figure 5.2 and Table 5.3, respectively). These results identified the treatment factor i.e. control and probiotic, as the main driver of the differences in the bacterial composition associated to the mucosa (weighted Unifrac Pseudo-F 9.63, p = 0.001), whereas the environment factor is driving the differences in bacterial community structure in the digesta (weighted Unifrac Pseudo-F 4.89, p = 0.012). Results from PERMANOVA analysis revealed an interaction between the treatment and environment factors in mucosa-associated microbiota (weighted UniFrac, Pseudo-F 5.02, p = 0.002). The latter is shown in the PCoA plots of mucosa samples where there is an evident separation between control and probiotic samples in seawater but not in freshwater. Significant differences between the control and the probiotic group in mucosa during seawater were consistent in both weighted and unweighted UniFrac suggesting that the bacterial community between both groups not only differ in the presence and absence of some bacteria but also in the relative abundance of some taxa. PCoA plots from unweighted UniFrac showed that microbial communities in the control samples from mucosa and digesta were clustered by the environment.

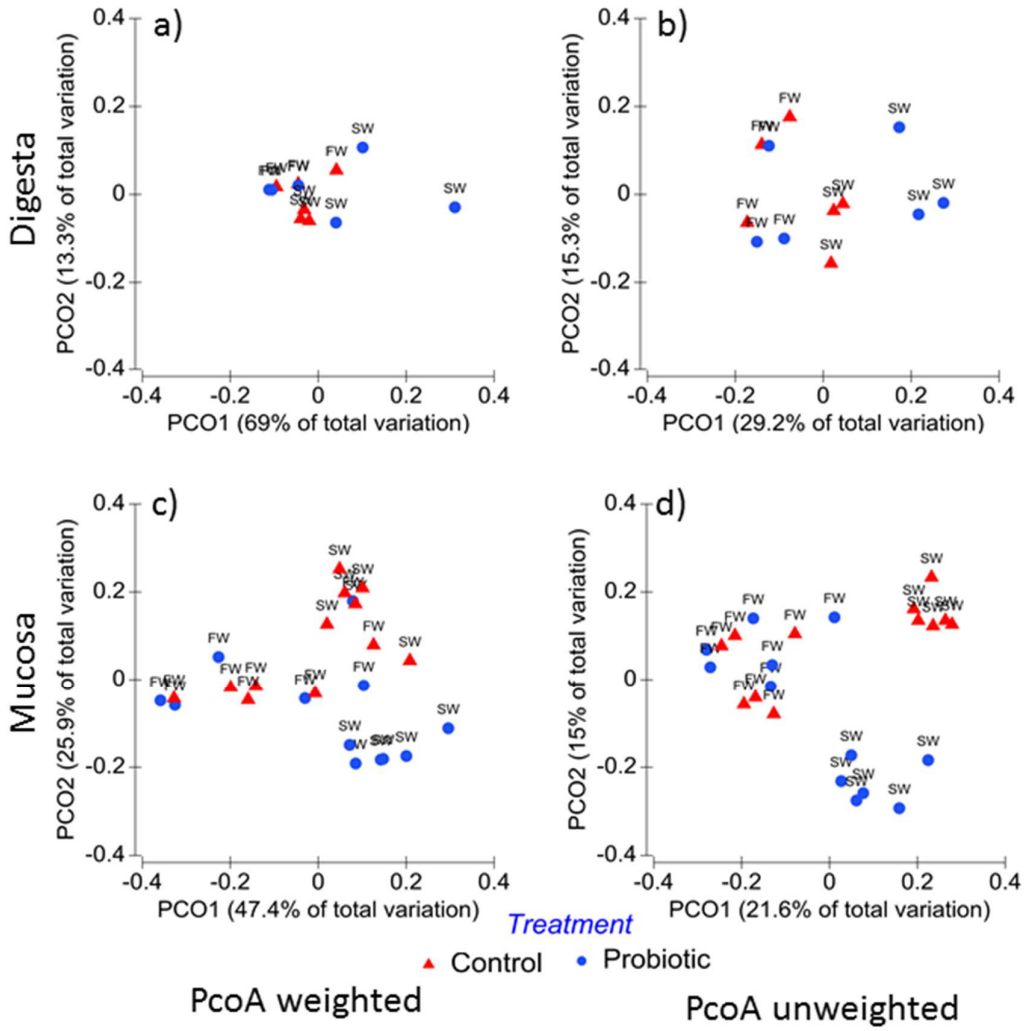


Figure 5.2. Principal coordinate analysis (PCoA) of the distal intestinal microbiota using UniFrac distances. The percentage of variation is explained by PC1 and PC2 axis. Each plot represents the differences between control (blue circles) and probiotic (red triangles) groups in both stages freshwater (FW) and seawater (SW). a) PCoA weighted digesta; b) PCoA unweighted digesta; c) PCoA weighted mucosa; d) PCoA unweighted mucosa.

Table 5.3. PERMANOVA results from weighted and unweighted UniFrac.

Group Comparison	PERMANOVA					
	Weighted Unifrac			Unweighted Unifrac		
	Average dissimilarity	Pseudo-F	*P*	Average dissimilarity	Pseudo-F	*P*
Digesta						
Treatment	0.16	1.10	0.322	0.39	1.22	0.208
Environment	0.19	4.89	0.012	0.42	3.38	0.003
Treatment x Environment		3.98	0.03		1.42	0.117
Mucosa						
Treatment	0.37	9.63	0.001	0.58	5.85	0.001
Environment	0.39	4.06	0.005	0.61	2.09	0.001
Treatment x Environment		5.02	0.002		2.86	0.001

High-throughput sequencing of region V1-V2 of 16S rRNA gene of the distal intestinal microbiota identified five phyla in digesta and eight in mucosa which accounted for more than 98% of the total abundance (Figure 5.3.). Digesta samples were strongly dominated by phylum Firmicutes mainly the classes Bacilli (>59%) and Clostridia (>6.2%), in all the experimental groups. Meanwhile, in the mucosa, the dominant groups varied according to the environment and treatment (Figure 5.3). During the freshwater stage the mucosa-associated bacterial microbiota of the control fed fish was dominated by Firmicutes (57%) followed by Proteobacteria (16.2%), whereas during the seawater stage the microbiota of the control fed fish was dominated by Firmicutes (27.8%), Fusobacteria (25.7%), Proteobacteria (19.5%) and Actinobacteria (17.6%). *Pediococcus* was identified by high-throughput sequencing in digesta and mucosa samples belonging to the treatment group in low abundance (<1%). The relative abundance of *Pediococcus* during the freshwater stage was 0.43% and 0.49% in digesta and mucosa respectively; meanwhile, the abundance of *Pediococcus* in the digesta and mucosa samples during the seawater stage was 0.14% and 0.027%, respectively. *Pediococcus* was also identified in the control group but at lower levels compared to the treatment group i.e. 3.4 times lower in digesta and mucosa during the freshwater stage and 1.45 and 2.05 lower in digesta and mucosa, respectively, during the seawater stage.

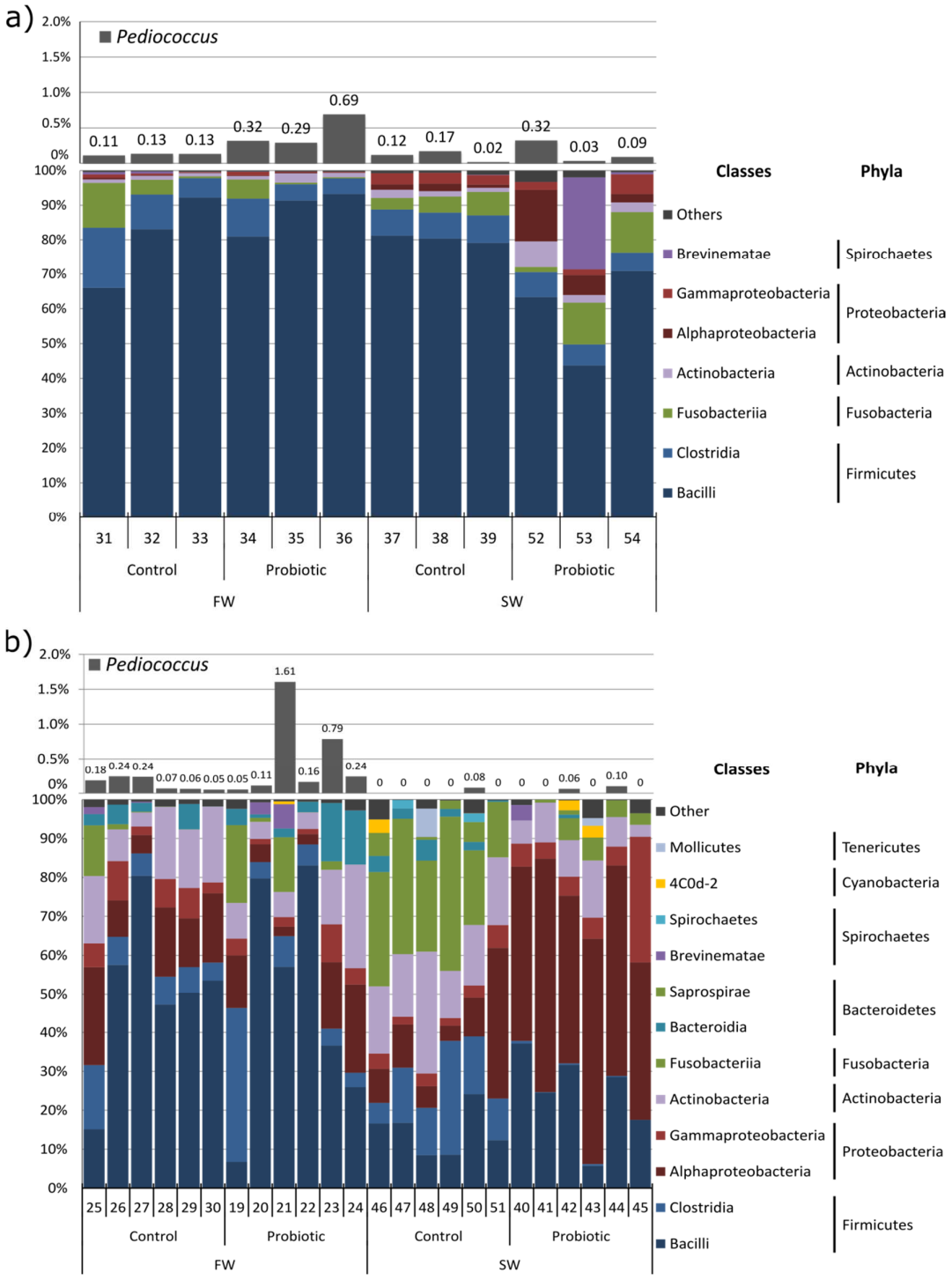


Figure 5.3. Relative abundance of bacterial communities at class and phylum level and abundance of the genus *Pediococcus* from a) Digesta and b) Mucosa of the distal intestine of Atlantic salmon fed control and probiotic diet during freshwater (FW) and seawater (SW) stages. Numbers below the bars represent the number of the tanks (a) or fish (b).

Linear discriminant analysis effect size (LEfSe) was used to identify the most important OTUs affected by the treatment (Figures 5.4., 5.5.). Overall, LEfSe results showed that the main differences between control and probiotic groups occurred in mucosa during the seawater stage. Fish fed the probiotic diet was associated with the enrichment of only a few OTUs in mucosa and digesta during the seawater stage. These OTUs belonged to the phyla Actinobacteria (family *Corynebacteriaceae*) and Proteobacteria (genera *Bradyrhizobiaceae* and *Herbaspirillum*). In contrast, a high number of OTUs was enriched in fish fed the control diet. In digesta, these OTUs belonged to the phyla Firmicutes (genus *Finegoldia*) and Proteobacteria (genera *Janthinobacterium* and *Sphingomonas*), meanwhile, in mucosa, the enriched OTUs belonged to the phyla Actinobacteria (genera *Micrococcus* and *Renibacterium*), Proteobacteria (genus *Haemophilus*) and Firmicutes (family *Streptococcaceae*, order Clostridiales and genus *Granulicatella*). To evaluate the effect of the environment on the specific members of bacterial microbiota associated to mucosa and digesta, samples from fish fed the control diet during freshwater stage were compared against control samples taken in the seawater stage with LEfSe (Figure 5.6. and 5.7.). These results identified a high number of OTUs that are differentially affected by the freshwater or seawater environment. Overall, the transfer to seawater had a higher significant effect on bacteria associated to mucosa than to digesta. Bacteria associated to digesta during the seawater stage were overrepresented by several OTUs belonging to the phyla Proteobacteria (classes Gammaproteobacteria, Betaproteobacteria and Alphaproteobacteria), Actinobacteria (genera *Dietzia*, *Mycetocola*, *Renibacterium*) and Cyanobacteria. During the freshwater stage, the bacterial digesta-associated microbiota was mainly enriched by the order Lactobacillales and the families *Coriobacteriia*,

Fusobacteriaceae, *Mogibacteriaceae*, *Halomonadaceae* and *Lachnospiraceae*. In the mucosa-associated microbiota, the phylum Firmicutes (class Bacilli) was enriched during the freshwater stage, whereas the phylum Fusobacteria (genera *Cetobacterium* and *Fusobacterium*) was significantly enriched during the seawater stage.

The core microbiota was analysed only in mucosa samples, due to the fact that digesta samples were pooled by tanks (Figure 5.8.). The core microbiota of both treatment groups during the freshwater and seawater stages had 14 shared OTUs, including 6 Proteobacteria, 3 Actinobacteria and 3 Firmicutes. The relative abundance of all the members of the core microbiota for each group varied from 12.8 ± 0.8 to 18 ± 2.2 %. Despite that, most of the member of the core microbiota belonged to Proteobacteria. Regarding abundance, Actinobacteria was the dominant group.

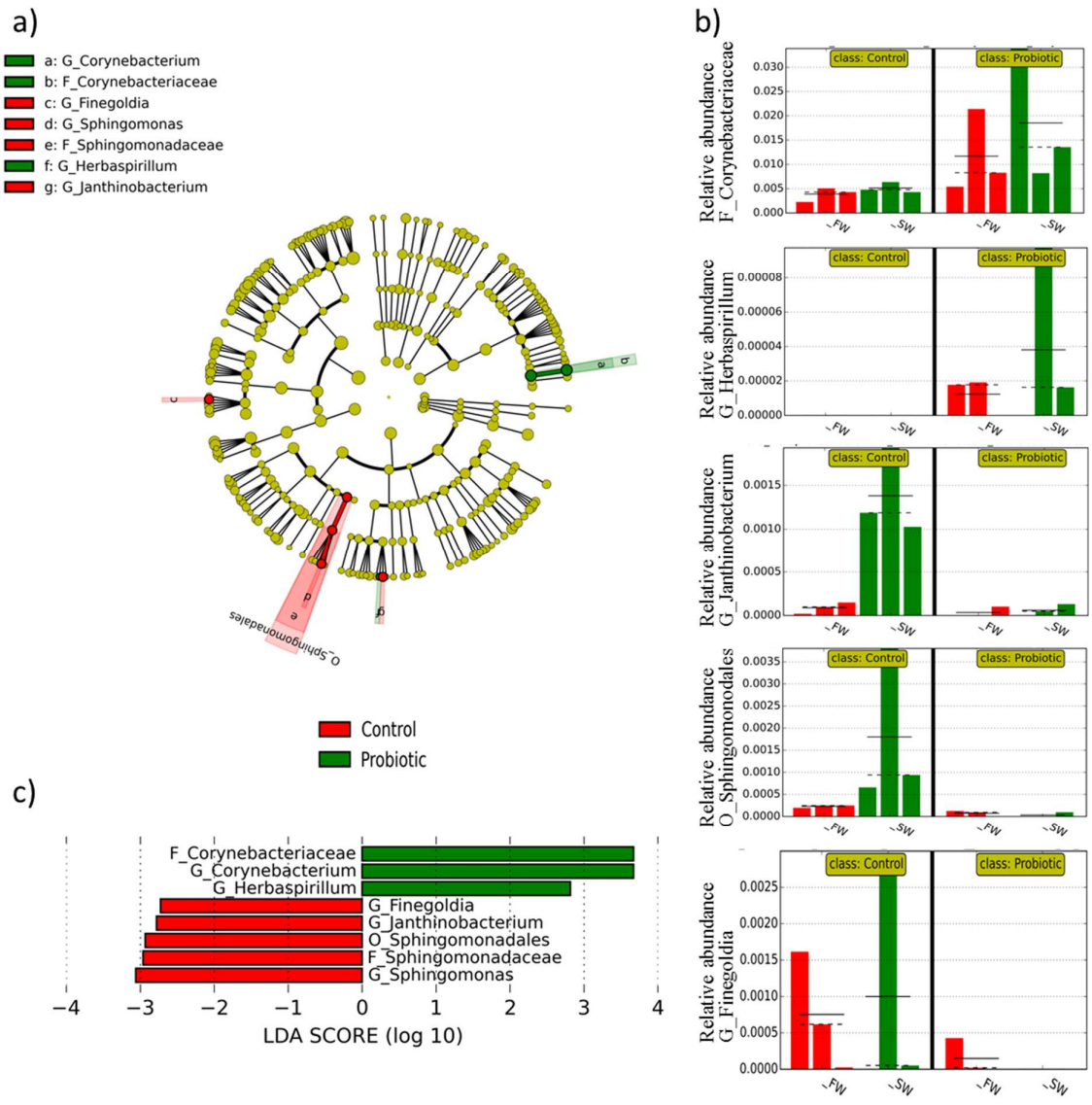


Figure 5.4. Taxonomic differences in distal intestinal microbiota from digesta between control and probiotic groups according to LEfSe analysis. The analysis was carried out with the relative abundance of all digesta samples at the genus level. Control and probiotic groups were treated as classes and freshwater (FW) and seawater (SW) stages as subclasses. a) A circular cladogram is representing the significant enriched OTUs between control (red) or probiotic (green) groups. No significantly different OTUs are represented in yellow. The diameter of each dots is proportional to its effect size. b) Relative abundance (expressed from 0 to 1) of enriched taxa according to LEfSe. When more than one OTU from the same phylogenetic clade was enriched according to LEfSe, only the relative abundance of the closest phylogenetic ancestor was plotted. c) Linear discriminant analysis (LDA), differentially enriched OTUs are arranged in descending order according to LDA score.

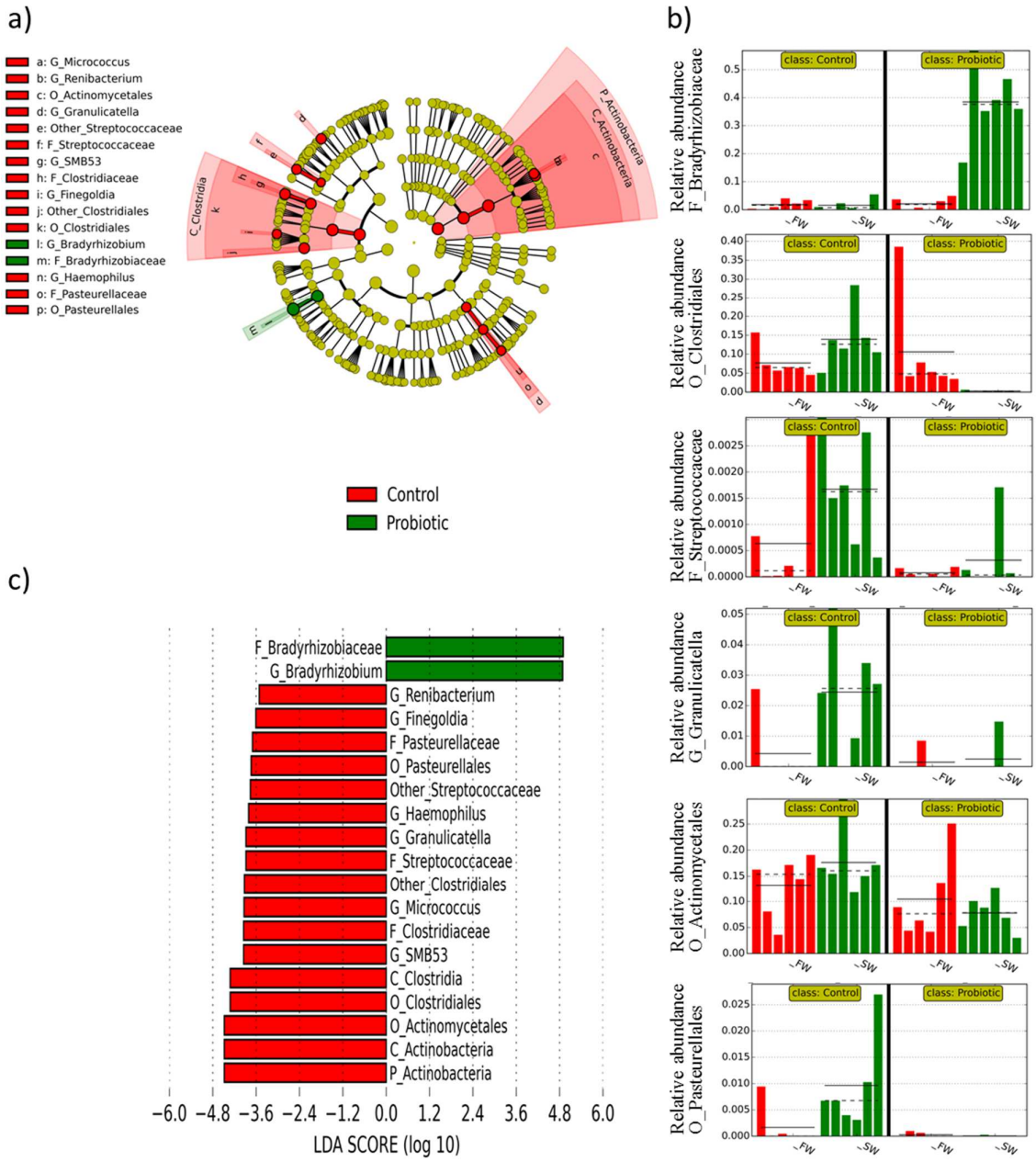


Figure 5.5. Taxonomic differences in the distal intestinal microbiota from mucosa between control and probiotic groups according to LEfSe analysis. The analysis was carried out with the relative abundance of all mucosa samples at the genus level. Control and probiotic groups were treated as classes, and freshwater (FW) and seawater (SW) stages as subclasses. a) A circular cladogram is representing the significant enriched OTUs between control (red) or probiotic (green) groups. No significantly different OTUs are represented in yellow. The diameter of each dot is proportional to its effect size. b) Relative abundance (expressed from 0 to 1) of enriched taxa according to LEfSe. When more than one OTU from the same phylogenetic clade was enriched according to LEfSe, only the relative abundance of the closest phylogenetic ancestor was plotted. c) Linear discriminant analysis (LDA), differentially enriched OTUs are arranged in descending order according to LDA score.

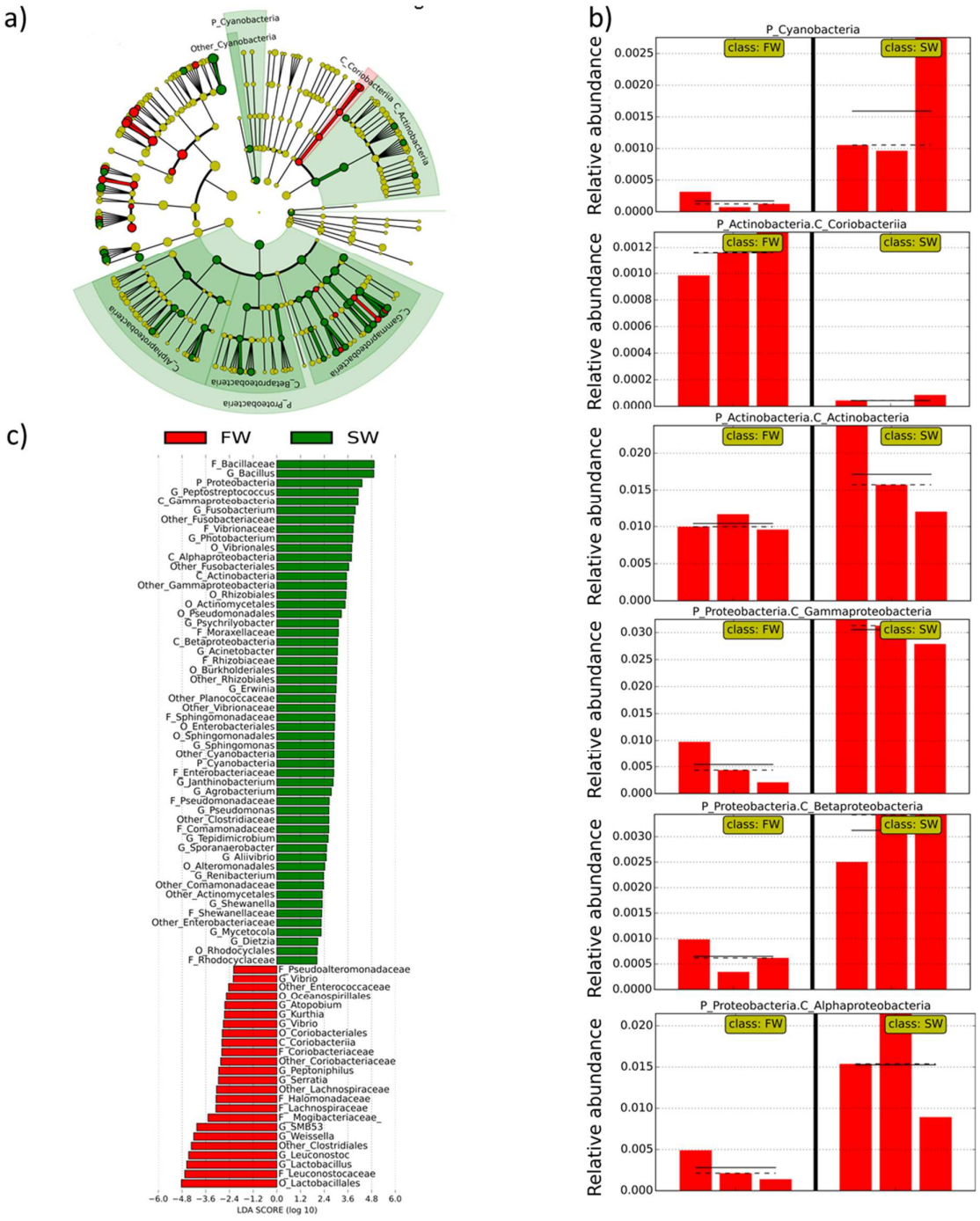


Figure 5.6. Taxonomic differences in the distal intestinal microbiota from digesta in control samples during freshwater (FW) and seawater (SW) stages according to LEfSe analysis. The analysis was carried out with the relative abundance at the genus level. The stage (FW and SW) was treated as a class. a) A circular cladogram is representing the significant enriched OTUs between FW (red) or SW (green) groups. No significantly different OTUs are represented in yellow. The diameter of each dot is proportional to its effect size. b) Relative abundance (expressed from 0 to 1) of enriched taxa according to LEfSe (only OTUs at class or phylum level were plotted). c) Linear discriminant analysis (LDA), differentially enriched OTUs are arranged in descending order according to LDA score.

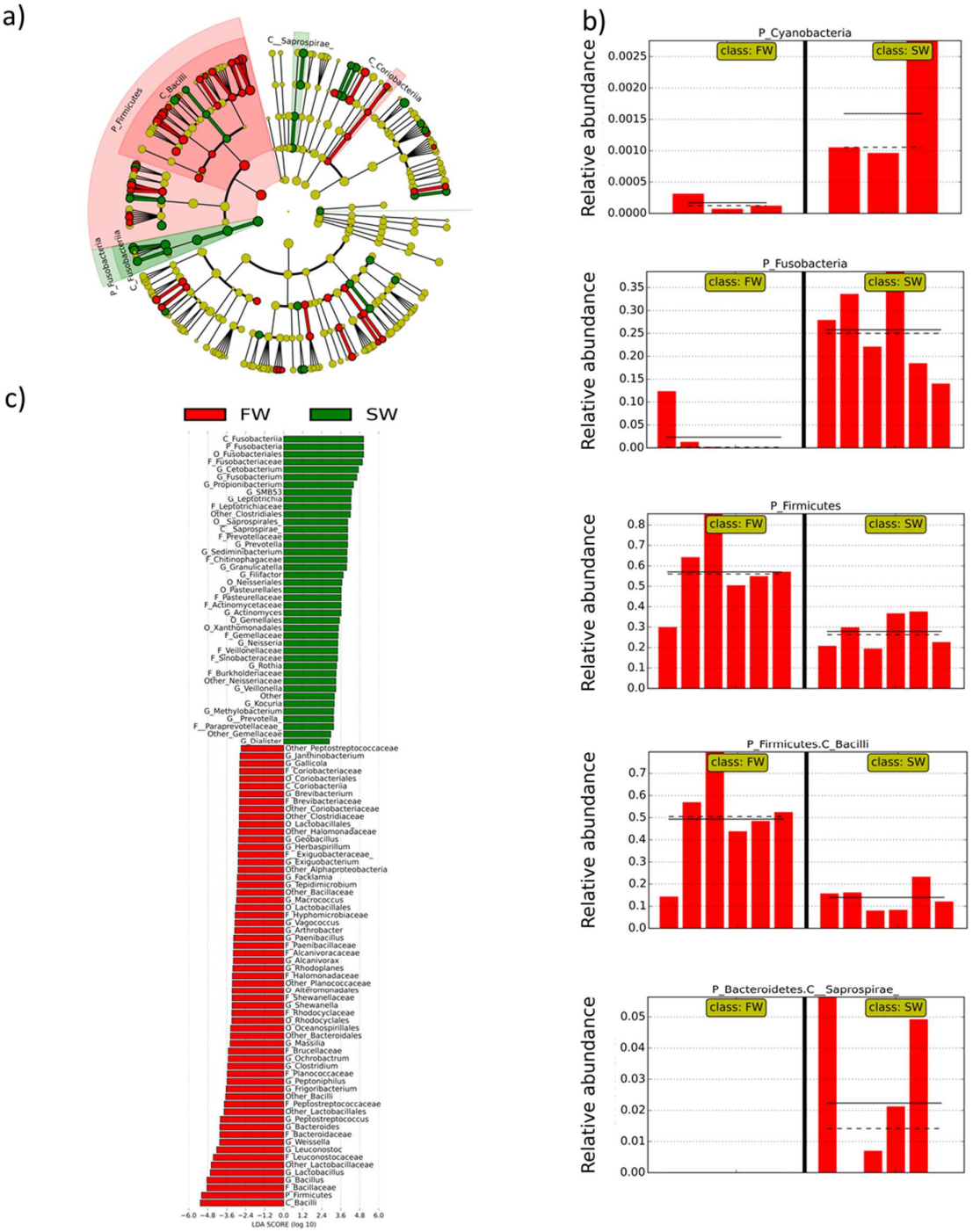
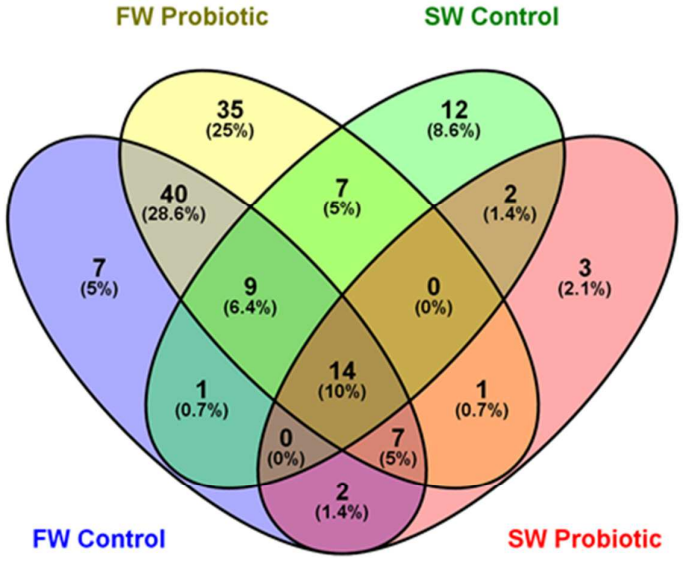


Figure 5.7. Taxonomic differences in the distal intestinal microbiota from mucosa in control samples during freshwater (FW) and seawater (SW) stages according to LEfSe analysis. The analysis was carried out with the relative abundance at the genus level. The stage (FW and SW) was treated as class. a) A circular cladogram is representing the significant enriched OTUs between FW (red) or SW (green) groups. No significantly different OTUs are represented in yellow. The diameter of each dot is proportional to its effect size. b) Relative abundance (expressed from 0 to 1) of enriched taxa according to LEfSe (only OTUs at class or phylum level were plotted). c) Linear discriminant analysis (LDA), differentially enriched OTUs are arranged in descending order according to LDA score.

a)



b)

Phylum	OTUs	FW Control	FW Probiotic	SW Control	SW Probiotic
Proteobacteria	F_Caulobacteraceae	0.03 ± 0.01	0.41 ± 0.1	0.1 ± 0.09	0.14 ± 0.45
	F_Other_Rhizobiales	1.38 ± 0.82	0.75 ± 0.84	5.05 ± 9.89	2.36 ± 0.05
	G_Ochrobactrum	0.86 ± 0.55	0.54 ± 0.26	0.18 ± 0.11	0.17 ± 0.02
	G_Other_Comamonadaceae	0.99 ± 0.73	0.49 ± 0.38	0.72 ± 0.96	1.03 ± 0.28
	F_Oxalobacteraceae	0.88 ± 0.89	1.07 ± 0.85	0.51 ± 0.3	0.45 ± 0.25
	G_Stenotrophomonas	0.3 ± 0.23	0.29 ± 0.36	0.3 ± 1.33	1.45 ± 0.78
Actinobacteria	G_Microbacterium	3.19 ± 2.69	2.14 ± 2.02	2.37 ± 0.95	1.71 ± 0.07
	G_Micrococcus	3.47 ± 1.75	2.19 ± 3.07	1.81 ± 0.6	0.27 ± 0.002
	G_Propionibacterium	2.26 ± 1.23	2.57 ± 3.49	7.04 ± 2.57	4.69 ± 2.64
Firmicutes	O_Other_Bacilli	1.03 ± 0.62	1.02 ± 0.61	0.2 ± 0.17	0.18 ± 0.01
	F_Other_Lactobacillales	1.48 ± 0.91	1.31 ± 0.52	0.38 ± 0.59	0.66 ± 0.32
	Total Abundance	15.9 ± 1.1	12.8 ± 0.8	18.8 ± 2.2	13.7 ± 1.3

Figure 5.8. Core microbiota of distal intestinal mucosa. a) Venn diagram showing the shared OTUs across 80% of the samples in all the experimental groups. Freshwater (FW), seawater (SW) stages.

5.4.2. Gene expression in the distal intestine

The expression of a panel of immune, stress and apoptotic related genes in the distal intestine was measured during FW and SW stages to evaluate the effect of the probiotic diet compared to the control diet, in the distal intestine of Atlantic salmon (Figure 5.9.). Genes related to antiviral protection were modulated in freshwater and seawater stages; *mx1* and *tlr3* levels were lower in fish fed the probiotic diet in the freshwater stage, but higher in the seawater stage in

comparison with fish fed control diet. Further, *pcna* and *tnfa* were significantly higher in the probiotic group than in the control group in the seawater stage. Higher expression of *il-1b* was seen in the fish fed the probiotic diet in contrast with the fish fed the control diet in the freshwater stage.

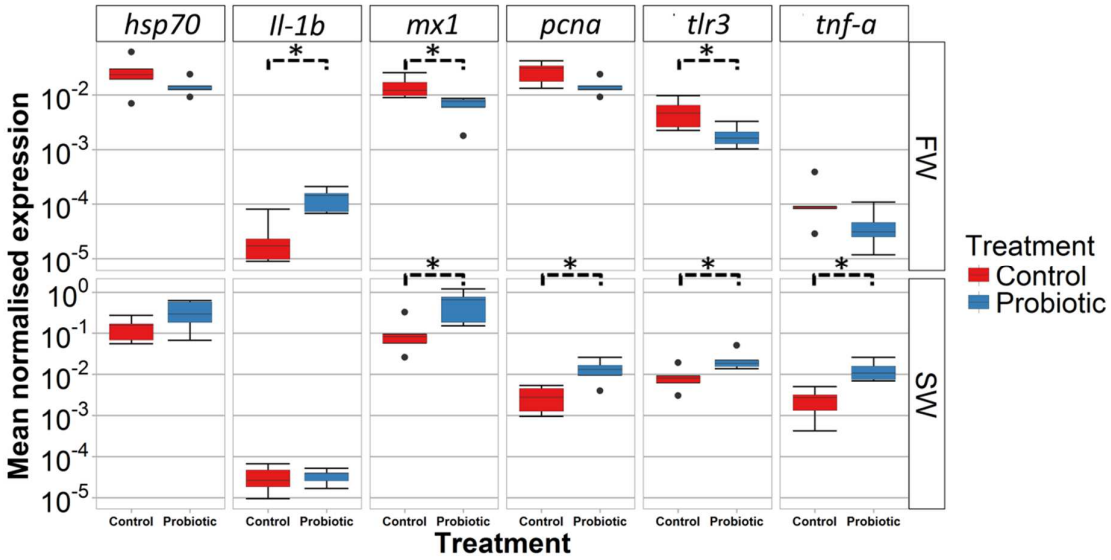


Figure 5.9. Gene expression profile of the distal intestine of Atlantic salmon fed control and probiotic diets during freshwater (FW) and seawater (SW) stages. Statistical differences between control and probiotic group (n = 5) are denoted by asterisks * (P < 0.05) and ** (P < 0.01).

5.4.3. Histology

The parameters evaluated by light microscopy were not significantly different between the control diet fed fish and the probiotic diet fed fish (Table 5.4.). The histological evaluation of distal intestinal morphology in control and probiotic groups during both environmental stages did not show any sign compatible with an active inflammatory response. The histological structure was characterised by a finger-like mucosa fold architecture, covered with an aligned epithelium of a single layer of enterocytes with supranuclear vacuoles in the apical zone, a thin lamina propria and low abundance of intraepithelial leukocytes.

Table 5.4. Histological parameters of the distal intestine of Atlantic salmon fed the experimental diets during freshwater and seawater stages. Data represent mean ± SD (n = 9).

	FW		SW	
	Control	Probiotic	Control	Probiotic
Mucosa fold length (µm)	322 ± 44	416 ± 58	518 ± 81	454 ± 106
Perimeter ratio (AU)	5 ± 1	6 ± 1	5 ± 1	6 ± 2
Goblet cells (per 100 µm)	16 ± 4	13 ± 3	16 ± 4	15 ± 3

FW – freshwater, SW – seawater, AU – arbitrary units

5.5. Discussion

In the last two decades, different studies using both dependent and independent-culture methods, have contributed to the characterization of bacterial microbiota in the intestine of Atlantic salmon as well as some basic understanding of the factors that may influence it (Spanggaard et al., 2000; Kim et al., 2007; Hovda et al., 2012; Navarrete et al., 2012; Zarkasi et al., 2014). Nonetheless, these studies have been focused on studying the microbiota during the marine stage of Atlantic salmon; in contrast, little information is available regarding the microbiota of Atlantic salmon during the freshwater stage. Moreover, this is the first known study that investigates the effect of the transition between freshwater to seawater stages on the intestinal microbiota on Atlantic salmon.

5.5.1. Effect of environment on distal intestinal microbiota

The use of molecular methods such as high-throughput sequencing has rapidly expanded our knowledge of the bacterial communities in the fish intestine (reviewed by Zhou et al. 2014). In the present study, the main phyla found in the distal intestine of Atlantic salmon during both freshwater and seawater stages were Firmicutes, Proteobacteria, Fusobacteria and Actinobacteria. Previous studies that investigated the intestinal microbiota of Atlantic salmon under farming conditions have also found these phyla as normal residents of the intestine (Zarkasi et al., 2014; Gajardo et al., 2016b; Schmidt et al., 2016; Dehler et al.). Despite heterogeneous experimental conditions and approaches between the present study and the studies reported by these authors, the phyla Firmicutes and Proteobacteria were consistently found as dominant bacteria in the intestine of Atlantic salmon. Although some of the phyla were consistently found in the

distal intestine of fish under the experimental conditions of this study, the transfer from freshwater to seawater had a major role in modulating specific bacterial communities associated to digesta and mucosa of the distal intestine as evidenced by the LEfSe analysis and alpha and beta diversity. However, the seawater transfer did not have the same impact on the bacteria associated to the digesta that it did on the bacteria associated to the mucosa, which was affected to a larger extent. The reasons for this are not yet clear. Information on the influence of different environmental factors on the intestinal microbial communities that investigated differences between bacteria associated to the digesta and the mucosa is scarce in fish.

In mammals, several authors have speculated that the mucosa-associated microbiota could have a stronger role in modulating the intestinal physiology than digesta-associated microbiota. This hypothesis is based on the fact that the mucosa-associated bacteria have a closer interaction with the host as they can influence the host both directly and indirectly than those in the digesta, which can only interact with the host indirectly (reviewed by Van den Abbeele et al. 2011). The latter would suggest that the intestinal microbiota shifting caused by transfer from freshwater to seawater could be involved in important physiological changes that occurred during the smoltification process of Atlantic salmon. The apparently stronger effect of the water environment in the mucosa-associated microbiota compared to the digesta-associated microbiota requires further investigation.

One of the main factors that may be responsible for influencing the intestinal microbiota during the transfer from freshwater to seawater is the salinity, which is a well-known factor that limits or promotes the establishment of specific bacterial communities in given environments (Lozupone and Knight, 2007;

Canfora et al., 2014). The effect of salinity on bacterial communities associated with fish has been previously studied (Schmidt et al., 2015; Lokesh and Kiron, 2016). A study conducted by Zhang et al. (2016) demonstrated that the bacterial community associated with the fish intestine also responds to salinity changes. In the present study, seawater environment significantly favoured the enrichment of *Fusobacterium* and *Cetobacterium* in the mucosa. These are anaerobic bacteria from the phylum Fusobacteria. *Cetobacterium* has often been reported to be part of the gut microbiota of freshwater fish and also reported as a species with potential to produce vitamin B12. Furthermore, Brugman et al. (2009) reported that a vancomycin treatment in zebrafish with enterocolitis increased the abundance of *Cetobacterium somerae* which was associated with a reduction of inflammation. On the other hand, *Fusobacterium* is rarely described in fish but often mentioned as part of oral and intestinal microbiota in humans (Chen and Jiang, 2015; D'Argenio and Salvatore, 2015). Some reports associate the presence of *Fusobacterium* spp. with different human pathologies (Kostic et al., 2012; Han, 2015). However, in fish, this bacterium has not been reported as a pathogen or correlated with an adverse effect in the intestine. The seawater environment was also responsible for a reduction of some lactic acid bacteria belonging to the phylum Firmicutes such as *Lactobacillus*, *Weissella* and *Leuconostoc* and for an increase of different members of phylum Proteobacteria. Information about the function of these bacteria in the intestine and the role in the adaptation of Atlantic salmon to seawater are unknown and require further investigation. Previous studies in salmonids have investigated the presence of an intestinal core microbiota (Wong et al., 2013; Gajardo et al., 2016b; Dehler et al.). In the present study, despite that the probiotic supplementation and the water environment had a significant impact on the microbiota associated with mucosa,

a core microbiota of 14 shared OTUs was identified across all the experimental groups. This set of “well-adapted” microorganisms account for approximately 15% of the total bacterial abundance. This suggests that the OTUs identified as members of this core microbiota are playing an important role in the distal intestine of Atlantic salmon. The presence of a relatively high number of OTUs belonging to the phylum Proteobacteria as part of the core microbiota of Atlantic salmon is in agreement with other authors (Gajardo et al., 2016b; Dehler et al.; Gajardo et al., 2017).

5.5.2. Effect of probiotic on distal intestinal microbiota

The use of probiotics in aquaculture has been implemented as a standard practice to improve the health and performance of the fish under stressful farm conditions (Lauzon et al., 2014; Pérez-Sánchez et al., 2014). Although the beneficial effect of some probiotics in humans and animals is well documented, there is still a lack of knowledge about the mode of action of these products. In aquaculture, this limitation is also evident. Some of the proposed possible modes of action of the probiotics are suggested to be mediated by the modulation of the microbiota (Merrifield and Carnevali, 2014; Wang et al., 2015). The present study investigated the effect of a dietary probiotic supplementation, namely *P. acidilactici,* in the distal intestinal microbiota. Currently, only two previous studies have investigated the role of *P. acidilactici* as a probiotic in the intestine of Atlantic salmon during the seawater stage (Abid et al., 2013; Vasanth et al., 2015). Both studies were able to identify a potential positive effect of this bacterium in the intestine of Atlantic salmon; however, only the study from Abid et al. (2013) investigated the effect of *P. acidilactici* on the intestinal microbiota. In the present study, the genus *Pediococcus* was detected by high-throughput sequencing in

digesta and mucosa samples. Overall, the abundance of this genus was low in both water environments, with lower abundance observed in the mucosa samples during the seawater stage compared to digesta samples during both the freshwater and saltwater stages and mucosa samples during the freshwater stage. Even though the study from Abid et al. (2013) did not include samples from freshwater, these authors did find a lower abundance of *Pediococcus* genus in mucosa compared to digesta samples in post-smolt Atlantic salmon using Bactocell® which is in line with the results observed in this study. Other studies using *P. acidilactici* as a probiotic in fish have demonstrated that this bacteria was able to survive in the intestine of freshwater (Ferguson et al., 2010; Merrifield et al., 2011) and seawater fish (Villamil et al., 2010; Lamari et al., 2013). In the present study, the *Pediococcus* genus was also detected in the intestine of fish fed the control diet but in lower levels than in fish fed the treatment diet. This genus has been reported previously as a normal inhabitant of the gut microbiota of Atlantic salmon (Merrifield et al., 2014; Gajardo et al., 2016b) and rainbow trout (Araújo et al., 2016). The difficulty in differentiating the native *Pediococcus* from the one used in the treatment diets as well as to classify this bacterium to species level are limitations of the approach used in the present study. Even though high-throughput sequencing has an improved resolution in comparison with other culture-independent methods such as DGGE and clone libraries, the high-throughput sequencing approach still rely on the taxonomic resolution power of the target gene and region sequenced, as well as the quality of the reference database used to assign the taxonomy. The latter and the limitations on sequencing length of current sequencing platforms reduce the confidence to discriminate between species of the same genus. More specific molecular methods such as PCR targeting alpha-subunit of phenylalanyl-tRNA synthase

and RNA polymerase genes have been suggested to differentiate species of the genus *Pediococcus* (Huys et al., 2012). Thus, further studies in this regard will be necessary to confirm and evaluate the survival of *P. acidilactici* in the distal intestine of Atlantic salmon during seawater stage.

Despite the low abundance of *Pediococcus* observed in mucosa samples during the seawater stage in fish fed the treatment diet, the beta diversity analysis showed that these samples had the highest modulation of the distal intestinal microbiota. Even though this finding is unexpected, results from high-throughput sequencing only give information regarding the relative abundance of each taxon. This information is dependent on the total bacterial population in the intestine, which was not evaluated in this study. Thus, a low relative abundance of highly metabolically active bacteria with probiotic properties could still be an important component of the community, with sufficient effect to produce a response in the host. Moreover, some of the benefits of probiotics may be mediated through mechanisms of action such as immune system activation by bioactive cell wall compounds that do not require the viability of the cell (reviewed by Lahtinen 2012).

5.5.3. Effect of probiotic on antiviral response

The effect of the commercial probiotic Bactocell® on the profile expression of a panel of immune, stress and apoptotic related genes on the distal intestine was measured in Atlantic salmon during freshwater and seawater stages. The evaluation of intestinal gene expression of *tlr3* and *mx1* was investigated in the present study due to the central role that these genes have in encoding proteins with antiviral response in Atlantic salmon (Arnemo et al., 2014; Caruffo et al., 2016).

The results of this study suggest that the supplementation of Bactocell® in the diet of Atlantic salmon modulates the intestinal antiviral response. This modulation was dependent on the environment as both investigated genes, namely *tlr3* and *mx1,* were significantly decreased during the freshwater stage, whereas a significant increase was observed during the seawater stage. This finding was unexpected and may suggest that mechanisms associated with the environment are influencing the antiviral response. One possible explanation for the different response of the intestine to probiotic supplementation could be related to the profound changes that take place during smoltification in Atlantic salmon. It is well known that the intestine plays a major role in the adaptation to the new marine environment. It is important to highlight that the strength of the modulation of *mx1* was higher during the seawater stage (5 times increased compared to the control group) than in the freshwater stage (2.2 times decreased compared to the control group). Studies have shown that *mx1* is expressed at high levels after the stimulation of *tlr3* agonist in head kidney leukocytes suggesting that these two genes are connected during the antiviral response (Arnemo, Kavaliauskis & Gjøen, 2014). These results are in agreement with Abid *et al.* (2013) who demonstrated up-regulation of *tlr3*, *tnf-α* and *mx-1* in the distal and proximal intestine of salmon in seawater stage (post-smolts) under a dietary regimen supplemented with *P. acidilactici* MA18/5M and short-chain fructooligosaccharides (scFOS) as symbiotic additives.

The main function of *tlr3* in innate immunity is to act as a sensor of viral RNA. High levels of expression of this gene have been detected in gut and spleen of salmon under physiological conditions (Arnemo, Kavaliauskis & Gjøen, 2014). Moreover, some authors suggest that the activation of *tlr3* could trigger the expression of genes encoding cytokines and proteins responsible for modulating

the immune response against viral infections (Rodriguez et al., 2005). Due to the important role of these genes in antiviral response, their increase in expression during seawater stage may suggest a potential protective role of Bactocell® in an eventual viral infection.

Activation of *tnf-α* and *il-1a* in the intestine are commonly associated with stimulation of the immune response. In the present study, fish fed the probiotic diet had a significantly higher response in *il-1a* and *tnf-α* compared to fish fed the control diet. However, this response was not consistent during the transfer to seawater. The activation of genes encoding pro-inflammatory cytokines in fish after supplementation with *P. acidilactici* has also been reported previously in Atlantic salmon (Abid et al., 2013) and tilapia (Standen et al., 2013). These authors suggested that activation of pro-inflammatory cytokines after supplementation with *P. acidilactici* may indicate a potential immunostimulatory response that could be beneficial to fight an eventual pathogen aggression. Future studies evaluating the response of Atlantic salmon to a specific pathogen are necessary to test the latter hypothesis.

Previous studies have investigated the expression of *hsp70* and *pcna* as markers for intestinal stress and cell proliferation in Atlantic salmon after adverse intestinal conditions (Olsvik et al., 2007; Sanden and Olsvik, 2009; Krogdahl et al., 2015). In this study, the low differences in the expression of both *hsp70* and *pcna* between the control and probiotic groups, together with a normal histological morphology of the intestine suggest that neither fish fed the control diet nor fish fed the treatment diet were undergoing an inflammatory response in the intestine.

5.5.4. Conclusion

Both factors evaluated in this study, i.e. dietary probiotic supplementation and transfer from freshwater to seawater, had a substantial impact on the microbial communities of the distal intestine of Atlantic salmon. However, it is important to highlight that this effect was more pronounced in the mucosa-associated microbiota. In both fish and mammals, it has been recognised that the digesta and mucosa compartments harbour substantially different microorganism (Eckburg et al., 2005; Looft et al., 2014; Gajardo et al., 2016b; Lyons et al., 2017). Thus, these two different microbial communities may have different roles in the intestine and may differently influence the health of the host. In fish, Most of the studies investigating factors that modulate the gut microbiota have focused on the so-called allochthonous microbiota, which is associated with faecal or digesta samples. The results found in the present study suggest that smoltification process in Atlantic salmon and a probiotic treatment affect in different extent the bacterial microbiota associated to digesta compared to the bacterial microbiota associated to the mucosa. As a consequence is suggested that sampling mucosa should be taken into consideration in future studies to have a better picture of the intestinal microbiota.

Changes of the bacterial microbiota in the mucosa observed in fish fed the treatment diet during seawater stage were associated with an activation of the antiviral response. The next Chapter will study genes related with antiviral response in other intestinal regions of Atlantic salmon fed a probiotic-supplemented diet during seawater stage as well as the effect on the intestinal microbiota. Furthermore, future studies should assess whether the potential activation of genes related to antiviral response, as observed in the present study,

may be related to a modulation of intestinal microbiota or other mechanisms associated with a marine environment and physiological changes during smoltification might be involved in the normal functionality of the intestine in Atlantic salmon. Studies including a viral challenge are also necessary to confirm if the antiviral response seen in this study by the probiotic bacteria *P. acidilactici* are reflected in a higher survival.

CHAPTER 6. Effect of fish oil replacement by rapeseed oil and dietary probiotic supplementation in the microbiota and intestinal health of Atlantic salmon

6.1. Abstract

The aim of the study was to characterise the microbiota of pyloric caeca and mid-intestine and to evaluate the modulatory effect of fish oil (FO) substitution by rapeseed oil (RO) alone or combined with *P. acidilactici* supplementation on the intestinal of Atlantic salmon. A 56-days-feeding trial was conducted in a seawater recirculation system. Fish received one of three diets: FO diet containing FO as the sole source of lipids, RO diet with a replacement of 70% of FO by RO and probiotic diet (RO-P) with the same formulation as RO but supplemented with *P. acidilactici*. Samples were taken from the pyloric caeca and mid-intestine mucosa for bacterial characterization. Intestinal health was evaluated by histology and gene expression. Specific growth rate (SGR), somatic indices and apparent digestibility (AD) of protein and lipids were also evaluated. No significant differences between treatments were evident in SGR, somatic indices and AD. The microbiota analysis showed differences in both alpha and beta diversity between the intestinal regions. The results from the beta diversity showed that samples clustered mainly by the intestinal region more than dietary treatments. The genus *Pediococcus* was not detected in any of the intestinal regions or the experimental groups evaluated. The FO replacement by RO or RO-P had a small impact on the pyloric caeca and mid-intestine expression profile of the genes evaluated in this study. The same trend was observed in the histological evaluation, with no changes in the parameters evaluated. The results of the present study showed that the replacement of FO with RO and the supplementation of RO with the probiotic *P. acidilactici* did not have a major impact on the intestinal health of Atlantic salmon. This study suggest that RO is a potential alternative for FO replacement.

6.2. Introduction

Salmonid aquaculture is a growing industry, thanks in part to the rising demand for high-quality seafood that cannot be met by supply from fisheries. The steady growing of the salmonid industry has been traditionally sustained on the use of marine ingredients. It is estimated that aquaculture uses the 87% of the world fish oil (FO) supply approximately and Atlantic salmon and rainbow trout production requires more than the 66% of the total FO used in aquaculture (reviewed by Turchini et al. 2009). However, the use of large quantities of marine ingredients is no longer ecologically nor economically sustainable, which has led to a gradual decrease in the use of FM and FO in salmon feeds and an increased use of alternative ingredients such as plant feedstuffs (Ytrestøyl et al., 2015).

Regarding FO replacement in diets for Atlantic salmon different vegetable oils (VO) have been used, including rapeseed oil (RO), olive oil, soybean oil, palm oil, linseed oil and capelin oil (Torstensen et al., 2005; Moldal et al., 2014). Rapeseed oil has been one of the preferred candidates for FO replacement in fish diets due to rapeseed is grown in different countries mainly in Europe and North America with good availability of non-genetic modified crops as well its good polyunsaturated fatty acid profile (Bell et al., 2003; Shepherd et al., 2017). Different VO have several components with potential physiological properties including antinutritional factors. Fish oil has high concentrations of DHA and EPA, whereas VO are rich in n-6 polyunsaturated fatty acids. n-6 polyunsaturated fatty acids are precursors of arachidonic acid, which has a central role in the inflammation. Atlantic salmon has specific requirements for DHA and EPA that may not be reached by the sole used of VO in the diets (Sissener et al., 2016),

which may lead to a detrimental effect on the overall health and welfare of the fish.

Most published research to date about the FO replacement by VO in diets for Atlantic salmon has primarily focused on studying growth performance and feed efficiency (reviewed by Turchini et al., 2009). Only a few studies have evaluated the effect of FO replacement by VO in intestinal health and microbiota of Atlantic salmon. A research conducted by (Moldal et al., 2014) concluded that feeding of fish with high inclusion of different VO was associated with shortened in the intestinal mucosa folds, and moderate alterations in the expression of immune-related genes in Atlantic salmon compared to the FO reference diet. Navarrete et al. (2012) showed that the intestine of rainbow trout fed with RO had lower bacterial richness compared with the fish fed FO. Moreover, studies in other fish species have observed that VO are able to modulate the microbiota of distal intestine compared to a reference diet based on FO (Torrecillas et al., 2017). Thus, additional research is needed to replace further or eliminate FO in Atlantic salmon diets.

The results of Chapter 3 demonstrated that the effect of the probiotic *P. acidilactici* in the microbiota of rainbow trout was modulated by plant ingredients in the diet. To the author's knowledge, the effect on the microbiota and intestinal health of Atlantic salmon after supplementing probiotics in diets with high inclusion of vegetable oil has not been studied.

Therefore, this Chapter aimed to: 1) characterise the differences in microbiota of pyloric caeca and mid-intestine, 2) evaluate the influence of FO substitution by RO and the supplementation of *Pediococcus acidilactici* in the intestinal microbiota, 3) determine the effect of FO substitution by RO and the

supplementation of *Pediococcus acidilactici* in overall health of Atlantic salmon in relation to histological parameters, gene expression profile and growth performance.

6.3. Materials and Methods

6.3.1. Animal husbandry

A 56-days-feeding trial was performed at the aquarium facilities of BioMar at Hirtshals (Denmark). A fish batch of 324 Atlantic salmon post-smolt initial weight of 173 g ± 5 were randomly allocated into nine 0.8 m^3 fibreglass tanks (36 fish per tank), containing 1000 L of sea water (33g/L ± 1 g/L). Throughout the experiment, the fish were kept in a seawater recirculation system with a continuous 24 h light photoperiod. During the experimental period, the dissolved oxygen level was maintained above 85%; the temperature was 15 ± 1 °C. The feeding trial was run in triplicate randomly allotting tanks for each experimental group.

6.3.2. Diets and experimental design

Three different diets were formulated to be iso-lipidic (20%) and iso-nitrogenous (50%). The reference diet (FO) contained fish oil as the main lipid source; the rapeseed diet (RO) and rapeseed probiotic diet (RO-P) were formulated to replace 70% of fish oil by rapeseed oil. The feed ingredients and chemical composition of the experimental diets are shown in Table 6.1. Two batches of RO diet were manufactured; one served as the control, and the other was supplemented with the probiotic Bactocell®. The recovery of *P. acidilactici* in the probiotic diets was 3,03 x 10^6 CFU/g The diets were produced by BioMar AD (Denmark). Fish were fed continuously by automatic belt feeders (8 hours feeding) and feed consumption was recorded daily.

Table 6.1. Formulation of the experimental diets and chemical composition

Ingredients (%)	Fish oil diet (FO)	Rapeseed diet (RO)	Rapeseed probiotic (RO-P)
Fishmeal	25.0	25.0	25.0
Soya protein concentrate	17.0	17.0	17.0
Wheat Gluten	12.0	12.0	12.0
Maize Gluten	5.0	5.0	5.0
Sunflower Expeller			
Horse Beans, Dehulled	8.1	8.1	8.1
Fish Oil	20.3	6.5	6.5
Rapeseed Oil		14	14
Wheat	8.5	8.3	8.3
Vitamineral mix	4.2	4.2	4.2
Bactocell®			0.03
Chemical composition (%)			
Moisture	7.2	7.3	7.3
Crude protein	44.3	44.3	44.3
Lipids	24.2	24.4	24.4
Ash	7.8	7.8	7.8
Gross energy (MJ kg^{-1})	19.5	19.5	19.5

All dietary ingredients were sourced from BioMar's routine suppliers (not listed here for commercial reasons.

6.3.3. Sample collection

After 56-days-feeding, 3 selected fish per tank were randomly taken, euthanised and sampled (n = 9). The intestines were aseptically removed using sterile instruments and divided into the proximal, mid and distal intestine. The whole pyloric caeca and the mid-intestinal mucosa from individual fish were collected into sterile tubes. The dissection and sampling methodologies used are described in section 2.3.

6.3.4. Growth performance

Increase in weight gain (WG) and specific growth rate (SGR), were calculated as described in Section 2.2

6.3.5. Microbiological analyses

For analysis of the pyloric caeca and mid-intestine microbiota, 3 fish per tank (*n* = 9) were used. The sampling was conducted as described in Section 2.3. and the analysis was conducted according to Section 2.4.

6.3.6. Gene expression analysis

Pyloric caeca and mid-intestine from 9 fish (3 fish per tank), were sampled for gene expression analysis. Each target gene was normalised using the geometric average expression of two reference genes, *elongation factor 1* and *gapdh* for the pyloric caeca and *elongation factor 1* and *beta-actin* for the mid-intestine. RNA extraction, cDNA synthesis, real-time PCR and data analysis were carried out as described in Section 2.5. The primer sequences of the genes evaluated in this study are presented in Table 6.2.

Table 6.2. List of primers used for gene expression.

Gene name	5'-3' primer sequence		Amplicon size (bp)	Annealing temperature (°C)	Primer efficiency	GenBank accession no.	Reference
	Forward	Reverse					
Permeability							
aqu-8ab	GGAGCTGCCATGTCAAAGAT	CGCCCCTAGCAATACTACCA	159	60	2.0	KC626879.1	(Kortner et al., 2012)
claudin-15	GGCACGTCTGAGAAACAACA	TAGGAAGTGGCAGCCTGACT	92	60	2.0	BK006395	(Tipsmark et al., 2010)
claudin-25b	CCTGTAAGAGGGGTCCATCA	TGACACATGTTCTGCCCTGT	101	60	1.9	BK006399	(Tipsmark et al., 2010)
occludin	GACAGTGAGTTCCCCACCAT	ATCTCTCCCTGCAGGTCCTT	101	60	2.1	NM_001173656.1	(Tipsmark and Madsen, 2012)
jam-1b	CGTTGCGGAAGGGCGTAG	CCAGCGATGTGTCCGATTTC	146	60	2.0	GBRB01043958.1	(Hu et al., 2016)
e-cadherin	ACTATGACGAGGAGGGAGGT	TGGAGCGATGTCATTACGGA	107	60	1.9	BT058864.1	(Hu et al., 2016)
Inflammation							
il-1b	GGACCTGCTCAACTTCTTGC	CTGTGATGTACTGCTGAACCC	112	60	2.1	NM_001123582.1	This study
anx-a1	GTCAGAATCTTGGTCCTGGTTC	ACTGCCGTAGTGAAGTGTGCT	98	60	1.9	CA060324.1	(Vasanth et al., 2015)
il-17a	CGAAGTACCTGGTTGTGTGC	TCCCTCTGATTCCTCTGTGG	143	60	1.9	XM_014193546.1	This study
tnf-a	ACACACTGGGCTCTTCTTCG	GCACTTGACCCTAAACGAAGC	52	60	2.0	NM_001123589.1	This study
Antiviral response							
ifn-a	ACTGAAACGCTACTTCAAGAAGTTGA	GCAGATGACGTTTTGTCTCTTTCCT	104	60	1.8	AY216595	(Wessel et al., 2015)
mx1	AAGCTGGCAGAGACACATGC	ACATCCTTTCTGCCGAGTCC	73	60	1.9	NM_001123693	This study
tlr-3	CTCTAACGGCAACCAGAAGC	ATGGTGAGGTTGGACAGAGG	144	60	1.9	BK008646	This study
Stress							
hsp70	TGGTCCTGGTGAAGATGAGG	TGGCCTGTCTCTGTGAATCG	108	60	2.0	AJ632154	This study
pcna	ACAGTTGTGTGGTCAGGATGC	GAACTTAACGCCATCCTTGG	110	60	1.9	BT056931	This study
Reference genes							
gadph	CCATCGCCAAGGTTATCAACG	TCTTCTGTGTGGCTGTGACG	84	60	1.9	XM_014141819.1	This study
efn-1	TCTTGGTCGTTTTGCTGTGC	AGCCTTGATGACACCGACAG	61	60	1.9	AF321836	This study
actin	TCAGGGAGTGATGGTTGGGA	GCCACTCTCAGCTCGTTGTA	170	60	1.9	XM_014194537.1	This study

6.3.7. Intestinal histology

Pyloric caeca and mid-intestine samples from 9 fish per tank were processed as described in Section 2.8.1. Each slide was digitalized at 40x magnification using a ScanScope AT Turbo slide scanner (Leica Biosystems, USA). The digital slides were examined using ImageScope Version 12.3 (Leica Biosystems, USA). Pyloric caeca and mid intestine were scored using six parameters, which were scored on a scale of 1 to 4 (Table 6.3.).

Table 6.3. Parameters and score used in the morphological evaluating of the pyloric caeca and mid-intestine.

Parameter	Score
Goblet cell frequency	1 = scattered cells 2 = increased number 3 = diffused number, multifocal 4 = tightly packed, highly abundant.
Intraepithelial leukocytes frequency	1 = absent 2 = mild 3 = moderate 4 = severe
Lamina propria width	1 = normal 2 = increased 3 = medium, clear increase in size 4 = severe increase in size, majority folds
Submucosa width	1 = normal 2 = focal mild increase in size 3 = clear increase in size 4 = extreme thick layer beneath many folds
Mucosal fold shrinkage	1 = absent 2 = focal stunting 3 = diffused stunting 4 = total tissue disruption
Hypervacuolated epithelium	1 = normal 2 = focal increase in size, moderate 3 = severe, diffuse vacuoles 4 = absence of supranuclear vacuoles

6.3.8. Statistical analysis

To evaluate the differences between the microbiota of pyloric caeca and mid-intestine, only fish fed the FO diet were analysed. To determine the effect of FO replacement in gene expression, as well as specific OTUs by LEfSe, fish fed the FO diet were compared against fish fed the RO diet. To determine the effect of probiotic diet on specific OTUs and gene expression fish fed the RO diet were compared against fish fed the RO-P diet. Statistical analysis was carried out using the methods described in Section 2.5.

6.4. Results

6.4.1. Growth, digestibility and somatic indices

The fish grew from an initial weight of 173 g ± 5 to an average of 420 ± 17 g during the trial period of 56 days (Table 6.3.). Specific growth rate ranged from 1.21 to 1.34. The fish fed the RO, and RO-P diets had better SGR, and apparent digestibility of crude protein and lipid than the fish fed FO diet. However, the differences were not significant. Dietary oil composition and probiotic supplementation had no effect on any of the somatic indices evaluated.

Table 6.4. Results of growth performance, lipid and crude protein apparent digestibility and somatic indices from Atlantic salmon fed the experimental diets for 56 days.

Parameter	FO		RO		RO-P	
	Average	SD	Average	SD	Average	SD
SGR	1.21	0.06	1.30	0.05	1.34	0.08
Initial weight (g)	172.4	7.6	176.9	4.8	170.1	3.1
Final weight (g)	400.7	56.6	427.8	65.7	432.2	75.6
Crude protein AD (%)	84.3	0.7	87.3	0.8	87.6	0.3
Lipid AD (%)	91.3	0.7	92.6	4.5	94.8	0.5
VSI	11.3	1.6	11.5	2.6	11.3	1.4
HIS	1.42	0.17	1.49	0.15	1.48	0.12
K	1.14	0.08	1.80	0.09	1.70	0.4

Fish oil - (FO); rapeseed oil - (RO); rapeseed oil probiotic - (RO-P); specific growth rate - (SGR); apparent digestibility- (AD); viscera-somatic index - (VSI), hepato-somatic index - (HIS); condition factor - (K)

6.4.2. Microbiota analysis

6.4.2.1. High-throughput sequencing data

Ion Torrent sequencing platform was used to sequence the variable region 1 and 2 of the 16S rRNA gene. A total of 3.0 million reads were generated from 54 samples sequenced before quality control. After quality filtering, processing the data in QIIME, filtering spurious sequences and discarding reads belonging to Streptophyta, a total

of 928,197 reads (17,188 ± 4,134 reads per sample) were retained. The percentage of removed reads affiliated to Streptophyta in mucosa samples of pyloric caeca and mid-intestine was 0.27% and 1.21%, respectively.

6.4.2.2. Intestinal microbiota

Alpha diversity parameters such as Chao1, phylogenetic diversity (PD) and Shannon index (Figure 6.1.) revealed significant differences between intestinal regions. Alpha diversity was significantly higher in the mid-intestine than in the pyloric caeca regardless the dietary treatment. Meanwhile, no significant differences were observed between fish fed the FO and RO diets or between fish fed RO and RO-P diet in any of the regions evaluated. The rarefaction curve based on the Chao 1 index reached the plateau, suggesting that the sequencing depth had a sufficient coverage to evaluate the bacterial diversity of both intestinal regions evaluated (mid-intestine and pyloric caeca).

The results from PCoA analysis revealed that the samples evaluated in this study clustered mainly by the intestinal region factor instead of the experimental diet (Figure 6.2.). This significant differences between the bacterial communities in the pyloric caeca and the mid-intestine were confirmed by PERMANOVA from both weighted and unweighted UniFrac. Even though the main factor leading the differences between bacterial communities was the intestinal region, minor but statistically significant differences were observed between the fish fed the FO diet, and the fish fed the RO diet according to unweighted UniFrac.

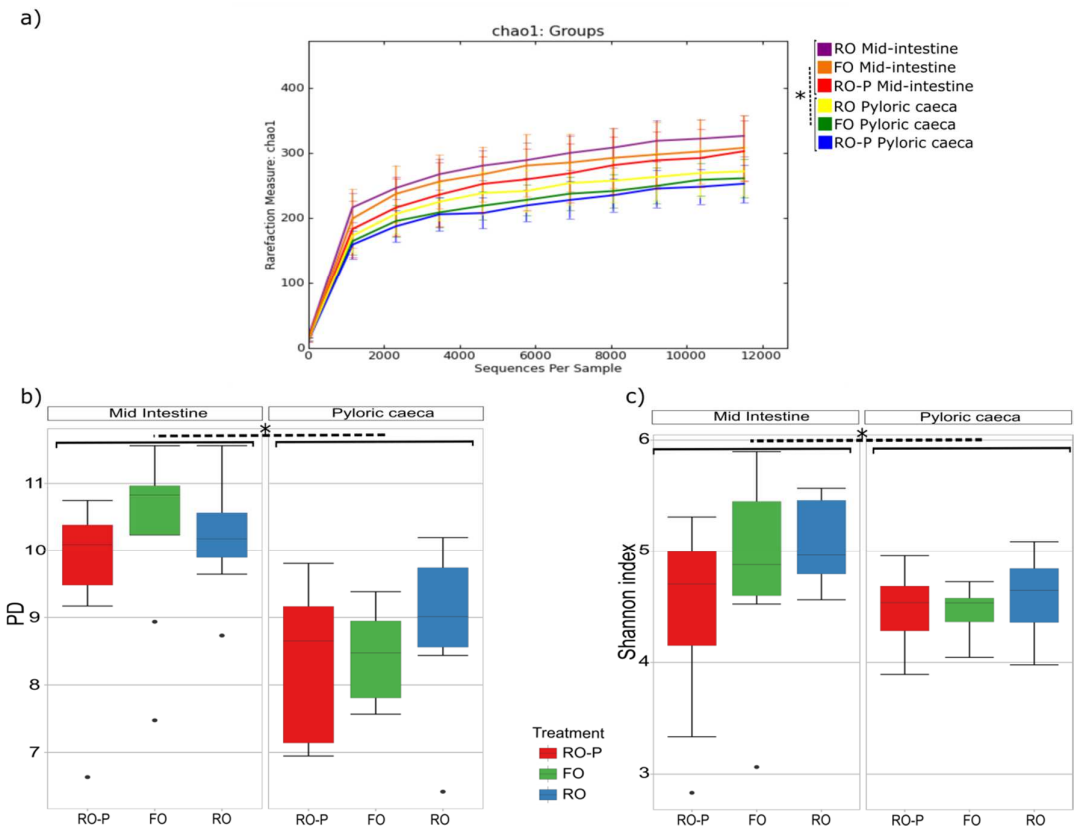


Figure 6.1. Alpha diversity parameters of the microbiota of the different experimental groups in the mid-intestine and pyloric caeca. Fish oil - (FO); rapeseed oil - (RO); rapeseed oil probiotic - (RO-P). a) Rarefaction curve based on Chao1 metric representing the average and standard deviation (error bars) of OTUs per experimental group; b) Phylogenetic diversity (PD) boxplot; c) Shannon index boxplot. Statistical analysis was conducted using alpha diversity parameters from samples rarefied at an even depth of 11,515 sequences. Statistical differences are denoted by asterisk * ($P < 0.05$).

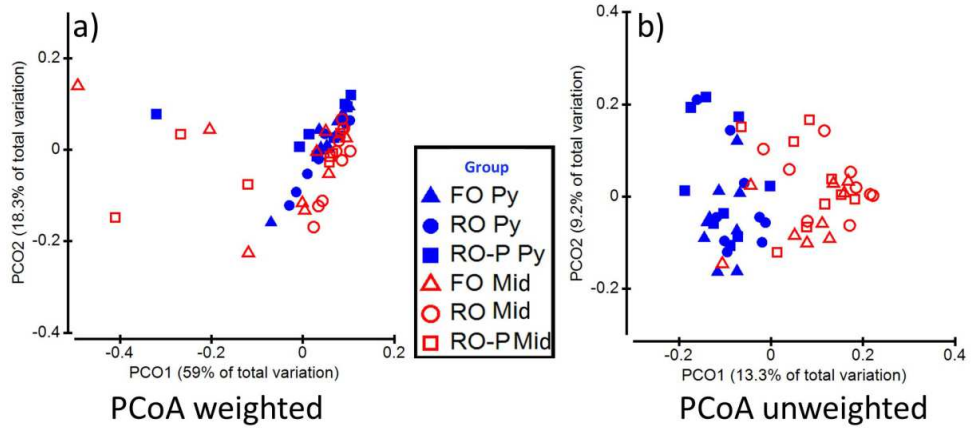


Figure 6.2. Principal coordinate analysis (PCoA) of the pyloric caeca and mid-intestine microbiota using UniFrac distances. a) PCoA weighted digesta; b) PCoA unweighted digesta. The percentage of variation is explained by PC1 and PC2 axis. Each plot represents the differences among intestinal regions pyloric caeca (Py, blue) and mid-intestine (Mid, red) as well as the different experimental groups, fish oil - (FO, triangles); rapeseed oil - (RO, circles); rapeseed oil probiotic - (RO-P, squares).

Tabla 6.5. PERMANOVA results from weighted and unweighted UniFrac.

Factor/Group comparison	PERMANOVA			
	Weighted UniFrac		Unweighted UniFrac	
	Pseudo-F/t	*P*	Pseudo-F/t	*P*
Diet	1.94	0.074	1.43	0.007
Pair-wise for Diet				
FO vs. RO	-	-	1.21	0.028
RO vs. RO-P	-	-	1.14	0.082
Intestinal region	4.15	0.008	5.85	0.001
Diet x Intestinal region	1.27	0.278	1.00	0.473

Fish oil - (FO); rapeseed oil - (RO); rapeseed oil probiotic - (RO-P).

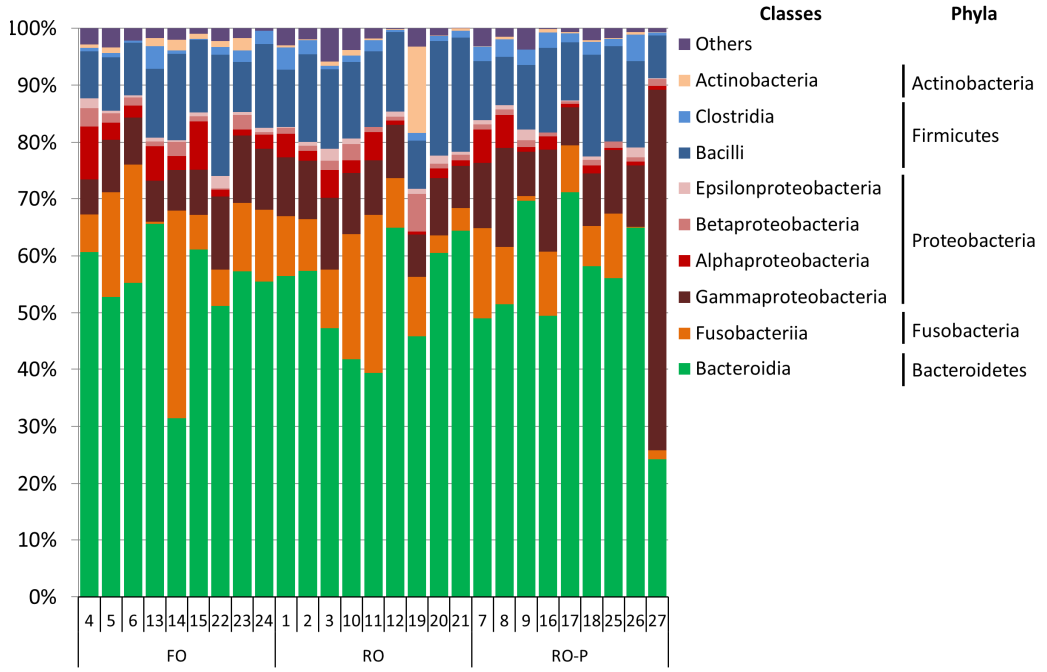
Bacterial characterization by HTS indicated that the microbiota from both intestinal regions evaluated belonged mainly (relative abundance >95%) to the same phyla and classes (Figure 6.3.). The most abundant phylum in the pyloric caeca and mid-intestine was Bacteroidetes. The second most abundant phylum was Proteobacteria mainly the class Gammaproteobacteria. The genus *Pediococcus* was not detected in any of the intestinal regions or the experimental groups evaluated.

To evaluate the differences in the microbiota between pyloric caeca and mid-intestine, LEfSe analysis was conducted comparing samples of both regions in fish fed the FO diet (Figure 6.4.). This analysis revealed that 27 taxa were significantly different between regions. The most remarkable difference between intestinal regions was the enrichment in the pyloric caeca of the phylum Bacteroidetes including the genus *Bacteroides*. The genera *Enterococcus*, *Arcobacter* and unidentified members of family *Enterobacteriaceae* and *Aeromonadaceae* were also significantly enriched in the pyloric caeca compared with the mid-intestine. On the other hand, a higher abundance of several taxa from the class Bacilli including the genera *Bacillus* and *Geobacillus* and members of the LAB group was detected in the mid-intestine compared to the pyloric caeca. The taxa from the LAB group enriched in the mid-intestine belong to the genera *Weissella and Streptococcus,* as well as unidentified members of the families *Bacillaceae* and *Leuconostocaceae*. Other taxa from the

classes Alphaproteobacteria, Betaproteobacteria and Gammaproteobacteria were also enriched in the mid-intestine, although to a lesser extent.

The effect of the FO replacement on specific taxa was evaluated by LEfSe in each intestinal region comparing the FO group against the RO group, whereas the effect of probiotic supplementation was assessed comparing the RO-P group against the RO group. Fish oil replacement and probiotic supplementation only produced few significant differences in OTUs of the pyloric caeca; only 6 and 8 OTUs were significantly modulated by the probiotic supplementation and FO replacement, respectively (Figures 6.5a and 6.5b). Most of the changes in specific taxa between experimental groups occurred in the mid-intestine. Probiotic supplementation induced changes in 12 different OTUs, while the RO diet-induced changes in 14 OTUs (Figures 6.5c and 6.5d). The mid-intestine of fish fed the RO diet had a significantly higher abundance of phylum Firmicutes compared with the fish fed the RO-P diet, including OTUs from the genera *Lactobacillus*, *Weissella* and *Lactococcus* as well as OTUs from Proteobacteria phylum including the genera *Arcobacter*, *Psychrobacter*, *Peptoniphilus* and an unidentified OTU from family *Halomonadaceae*. Regarding the mid-intestinal microbiota of fish fed the FO diet compared with fish fed the RO diet, the main changes involved the phylum Bacteroidetes (genus *Bacteroides*) and the genus *Lactococcus,* which were significantly less abundant in the FO group. In contrast, the genera *Cetobacterium*, *Plesiomona* and *Trabulsiella* as well as unidentified OTUs from family *Bacilli*, and classes ZB2 and Armatimonadia were enriched in the FO group.

a)



b)

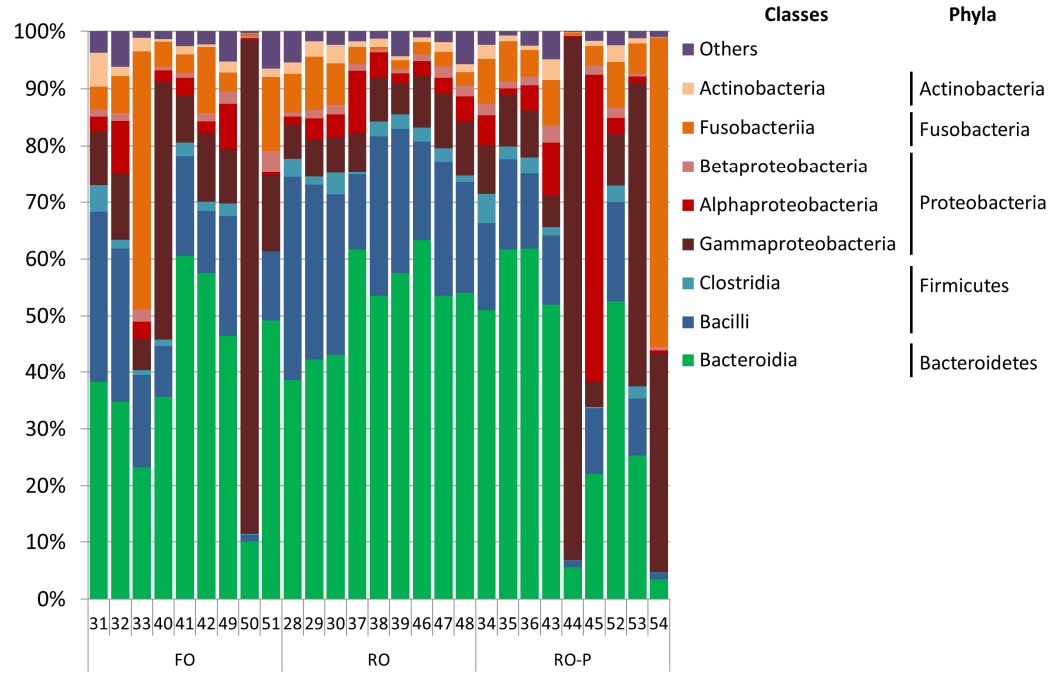


Figure 6.3. Relative abundance of the main bacterial taxa at class and phylum level for a). Pyloric caeca and b). Mid-intestine. Classes below an abundance average of 0.8% per experimental group are not shown but summarised in a mixed group "Others". Numbers below the bars represent the number of the fish for each experimental group. Fish oil (FO), rapeseed oil (RO) and rapeseed oil probiotic (RO-P).

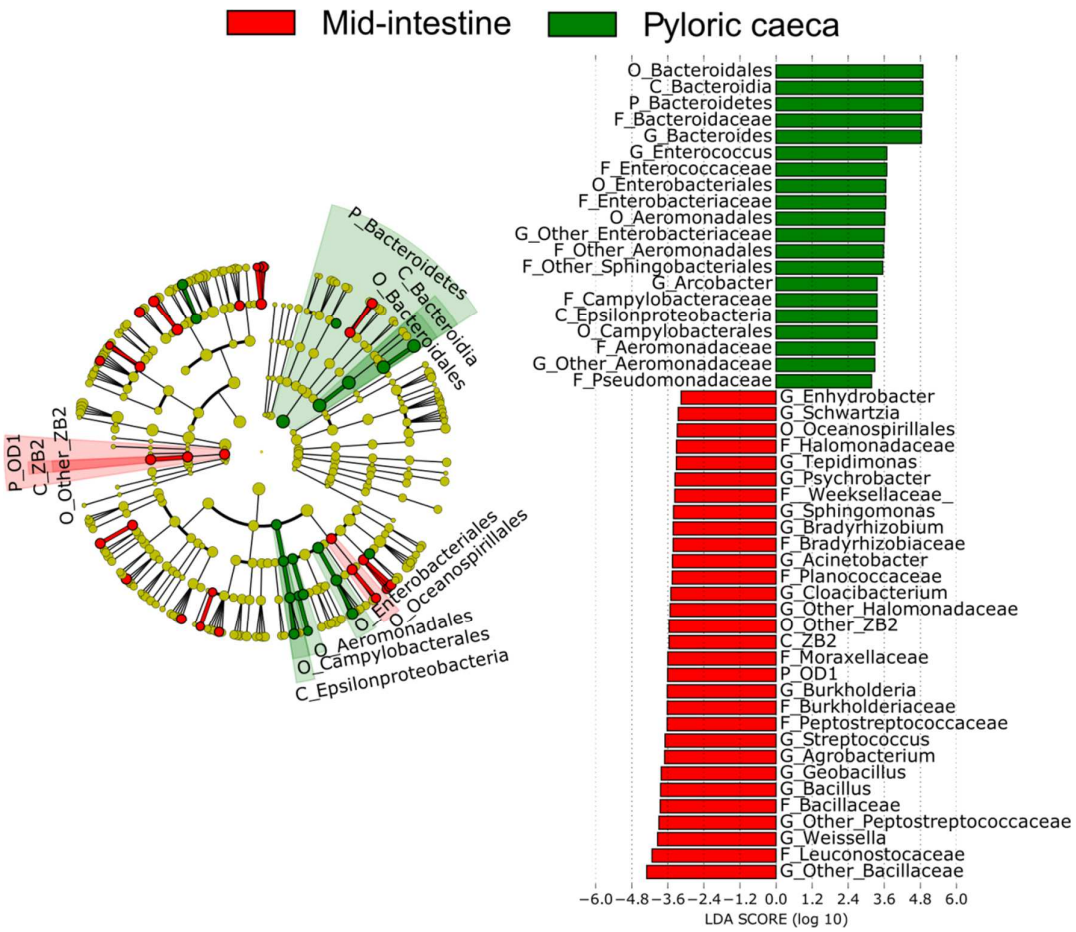


Figure 6.4. Taxonomic differences in the microbiota between pyloric caeca and mid-intestine according to LEfSe analysis. The analysis was carried out with the relative abundance of individual fish for each experimental group at the genus level. A circular cladogram (left) is representing the significant enriched OTUs for each respective group (red and green dots). No significantly different OTUs are represented in yellow dots. The diameter of each dot is proportional to its effect size. Linear discriminant analysis (left) represents the differentially enriched OTUs arranged in descending order according to LDA score).

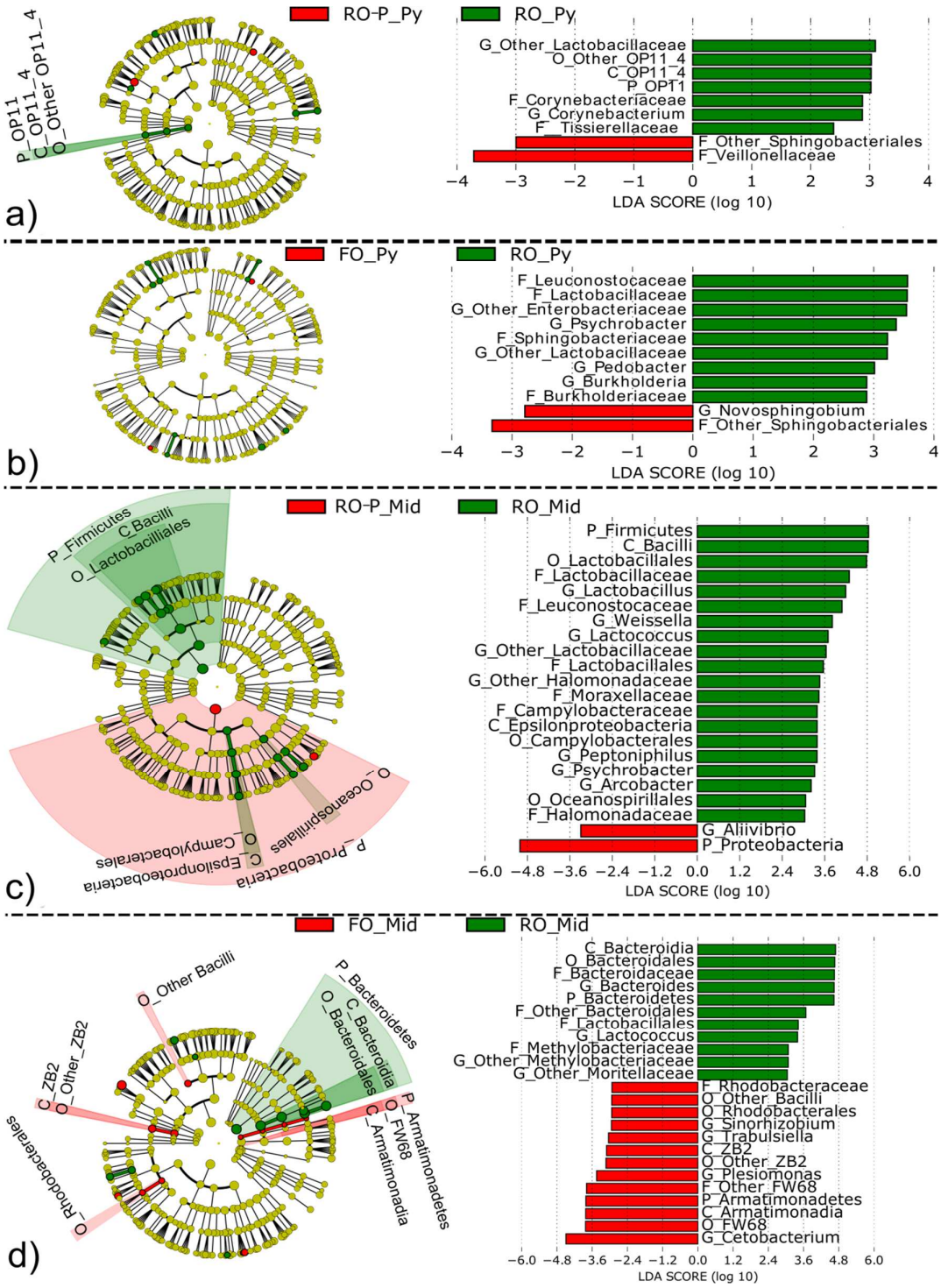
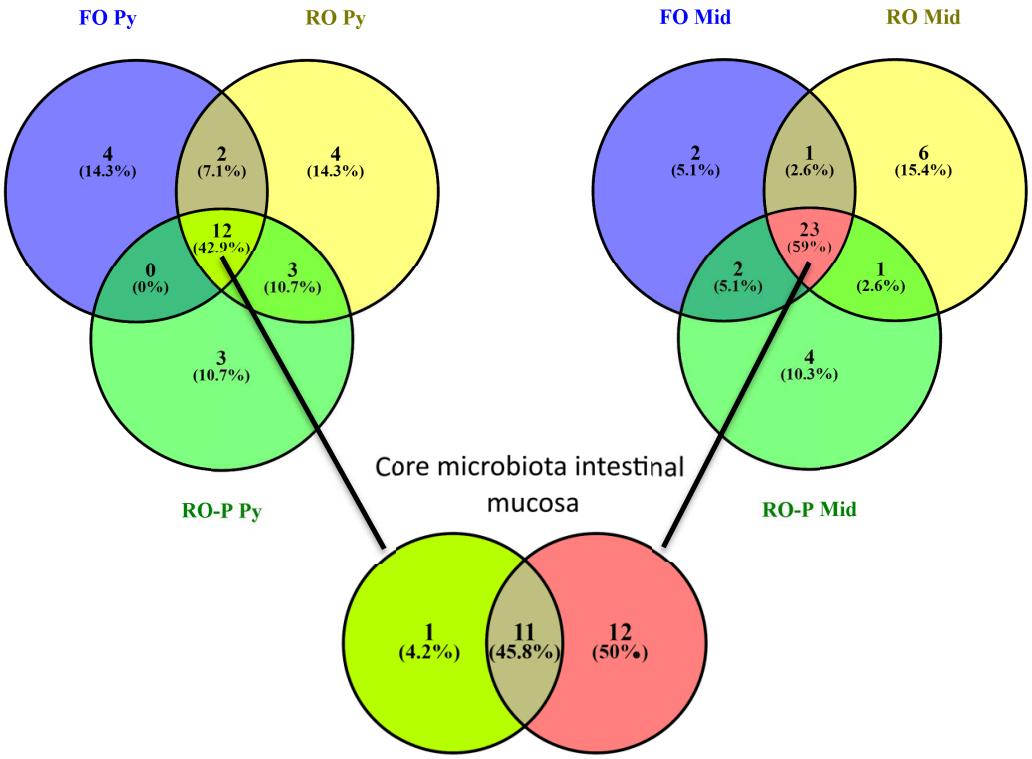


Figure 6.5. Taxonomic differences in the microbiota among experimental groups according to LEfSe analysis. The analysis was carried out with the relative abundance of individual fish for each experimental group at the genus level. A circular cladogram (left) is representing the significant enriched OTUs for each respective group (red and green dots). No significantly different OTUs are represented in yellow dots. The diameter of each dot is proportional to its effect size. Linear discriminant analysis (left) represents the differentially enriched OTUs arranged in descending order according to LDA score. Pyloric caeca - (Py), mid-intestine - (Mid), fish oil - (FO); rapeseed oil - (RO); rapeseed oil probiotic - (RO-P).

Figure 6.6. displays the core microbiota of pyloric caeca and mid-intestine with the contribution of each taxon in terms of relative abundance. The core microbiota of pyloric caeca was composed by 12 OTUs, which together accounted for 78.6% 83.7% and 80.6% of the relative bacterial abundance for the RO, RO-P and FO groups, respectively. The core microbiota of the mid-intestine was composed by 23 OTUs, which accounted for 79.9%, 81.7% and 83.9% of the relative bacterial abundance for the RO, RO-P and FO groups, respectively. In both, pyloric caeca and mid-intestine, the most dominant phylum of the core microbiota was Bacteroidetes, specifically the genus *Bacteroides*. The members of the core microbiota of pyloric caeca were stable to the influence of the FO replacement, and probiotic supplementation since no significant differences regarding relative abundance were observed among the experimental groups. On the contrary, several members of the core microbiota of the mid-intestine were significantly different among the experimental groups. For example, the taxa belonging to the genera *Weissella*, *Psychrobacter*, *Arcobacter* and an unidentified member of family *Halomonadaceae* were significantly lower in fish fed the RO diet than in fish fed the RO-P diet according to LEfSe. Furthermore, few significant differences in members of the core microbiota of mid-intestine were also observed between the FO and RO group. In consequence, the genus *Cetobacterium* was significantly higher in the FO group compared to the RO group. Meanwhile two OTUs belonging to the order Bacteroidales (genus *Bacteroides* and an unidentified OTU from the family *Bacteroidales*) were significantly lower in the FO group than in the RO group.

a) Pyloric caeca Mid-intestine



b)

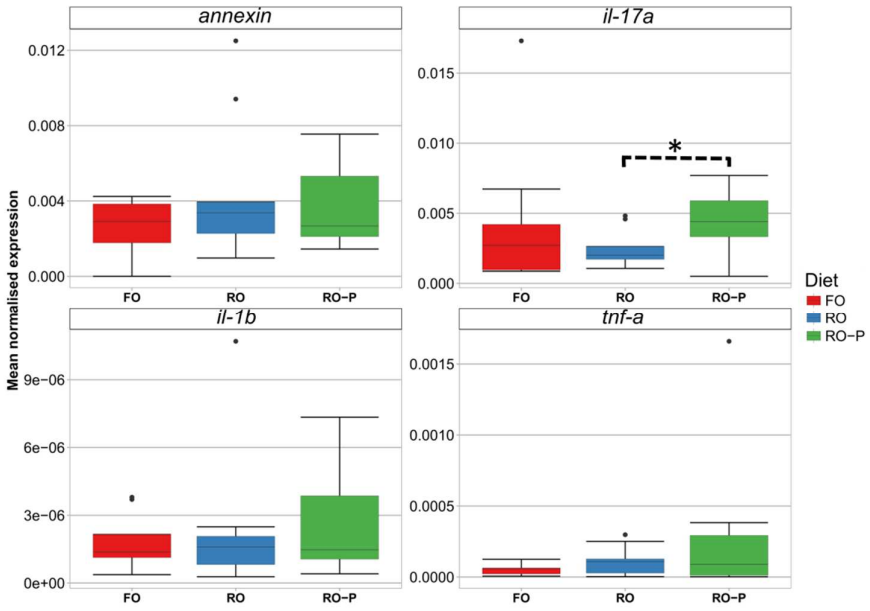
Core Taxonomy		Pyloric caeca core				Mid-intestine core			
Phylum	Order	Number of OTUs in the	Average abundance (%) RO Py	RO-P Py	FO Py	Number of OTUs in the	Average abundance (%) RO Mid	RO-P Mid	FO Mid
Actinobacteria	Actinomycetales	-	-	-	-	1	0.99	1.06	0.84
Bacteroidetes	Bacteroidales	2	53.11	54.91	54.49	2	51.91	37.16	39.45
	Flavobacteriales	-	-	-	-	1	0.52	0.33	0.60
Firmicutes	Bacillales	1	-	-	-	1	1.61	0.80	1.29
	Lactobacillales	2	9.29	9.92	8.85	3	12.95	6.97	9.88
Fusobacteria	Fusobacteriales	1	9.89	5.94	11.06	2	3.71	9.81	9.80
Proteobacteria	Rhizobiales	-	-	-	-	1	0.58	0.56	0.60
	Sphingomonadales	-	-	-	-	1	0.43	0.35	0.21
	Burkholderiales	-	-	-	-	2	0.42	0.38	0.45
	Campylobacterales	1	0.78	0.54	0.82	1	0.50	0.34	0.33
	Aeromonadales	2	0.78	0.81	1.11	2	0.21	0.60	0.36
	Alteromonadales	-	-	-	-	1	2.42	1.94	2.59
	Enterobacteriales	1	3.13	3.30	2.52	1	2.04	1.44	1.74
	Oceanospirillales	-	-	-	-	1	0.23	0.06	0.21
	Pseudomonadales	1	1.04	0.95	0.92	2	1.26	0.92	1.18
	Vibrionales	1	0.54	7.28	0.77	1	0.23	18.93	14.38
	Total	12	78.56	83.66	80.53	23	79.99	81.67	83.92

Figure 6.6. Core microbiota of pyloric caeca and mid-intestine. a) Venn diagram showing the shared OTUs across 80% of the samples per experimental group and core microbiota of intestinal mucosa. b) The contribution of each component of the core microbiota (average abundance) in each experimental group. Fish oil - (FO); rapeseed oil - (RO); rapeseed oil probiotic - (RO-P), pyloric caeca (Py) and mid-intestine (Mid).

6.4.3. Gene expression

The expression of a panel of genes related to inflammation, stress, antiviral response and permeability were measured in the two intestinal regions i.e. pyloric caeca and mid-intestine, to evaluate the effect of FO replacement and probiotic supplementation in Atlantic salmon. Results regarding gene expression in pyloric caeca are presented in Figure 6.7. and Figure 6.8., meanwhile the gene expression profiles for mid-intestine are displayed in Figure 6.9. and Figure 6.10. Overall, the FO replacement had a small impact on the pyloric caeca and mid-intestine expression profile of the genes evaluated in this study. Only levels of *e-cadherin* in the pyloric caeca and *occludin* in the mid-intestine were higher in fish fed the FO diet compared with fish fed RO diet. The FO group had a similar gene expression pattern to the RO group of genes related to stress, inflammation and antiviral response. When the gene expression profile of RO group was compared to RO-P group a modulation of some genes related to permeability, antiviral response and inflammation were observed in the pyloric caeca. Thus, fish fed the RO-P diet had higher levels of *il-17a*, *mx1*, and *claudin-25* in the pyloric caeca. *Occludin* level was also higher in RO-P group compared to RO group in the mid-intestine.

a)



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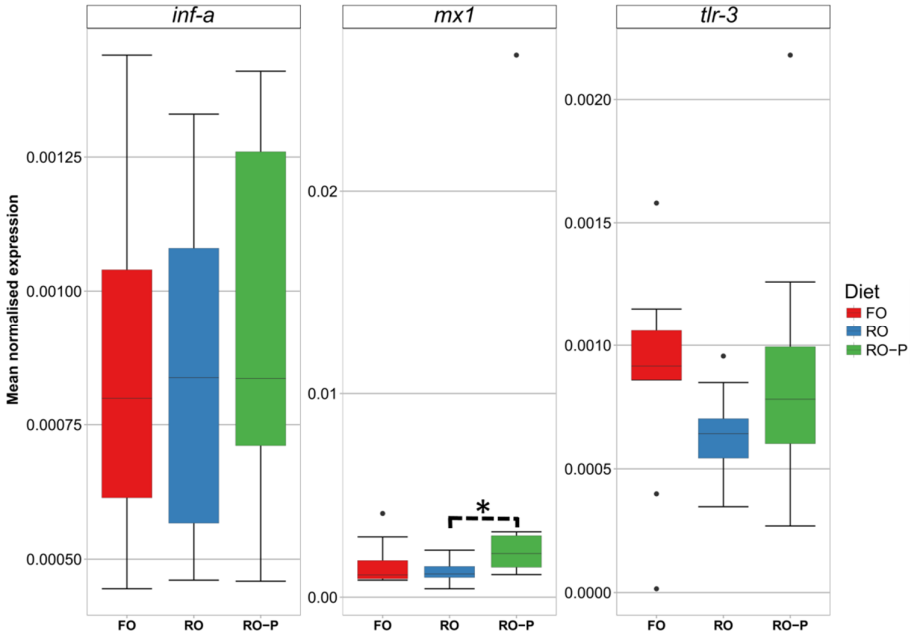
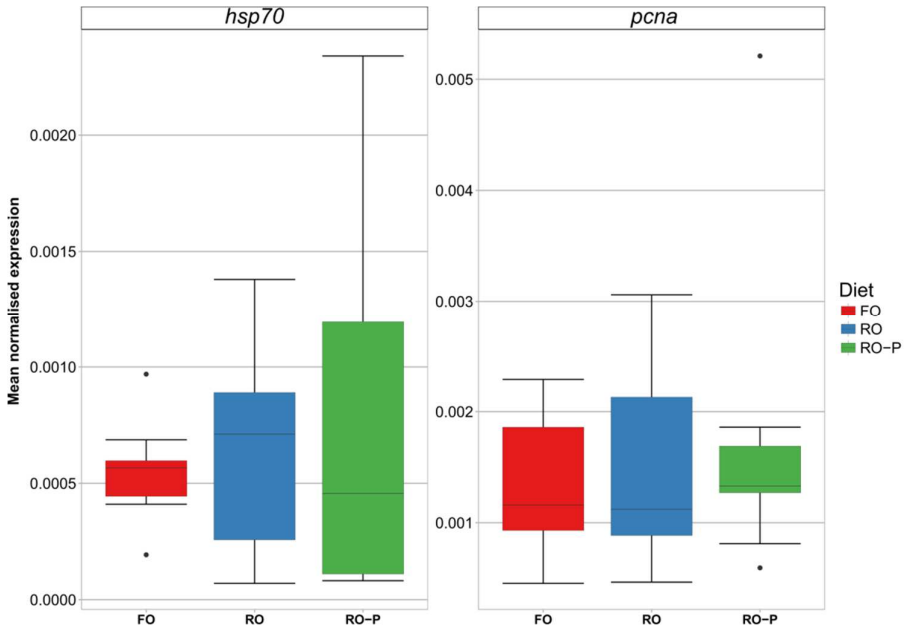


Figure 6.7. Expression profile of genes related to inflammatory and antiviral responses for pyloric caeca of Atlantic salmon fed the experimental diets. a) Inflammatory response and b) Antiviral response. Fish oil - (FO); rapeseed oil - (RO); rapeseed oil probiotic - (RO-P) Statistical differences between FO and RO or RO and RO-P (n = 9) are denoted by asterisks * (P < 0.05).

a)



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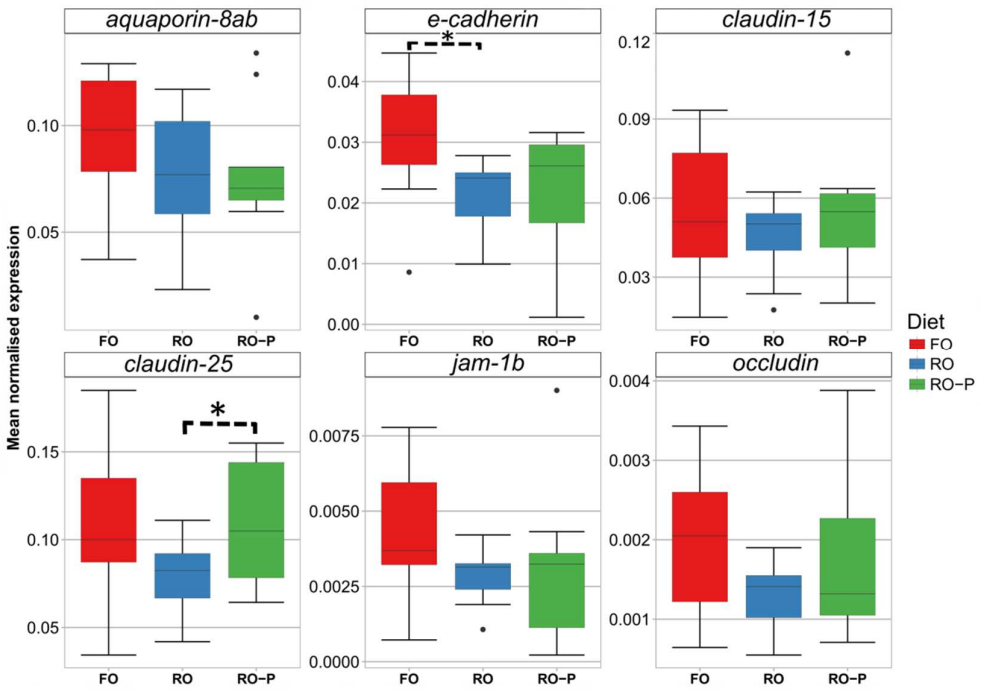
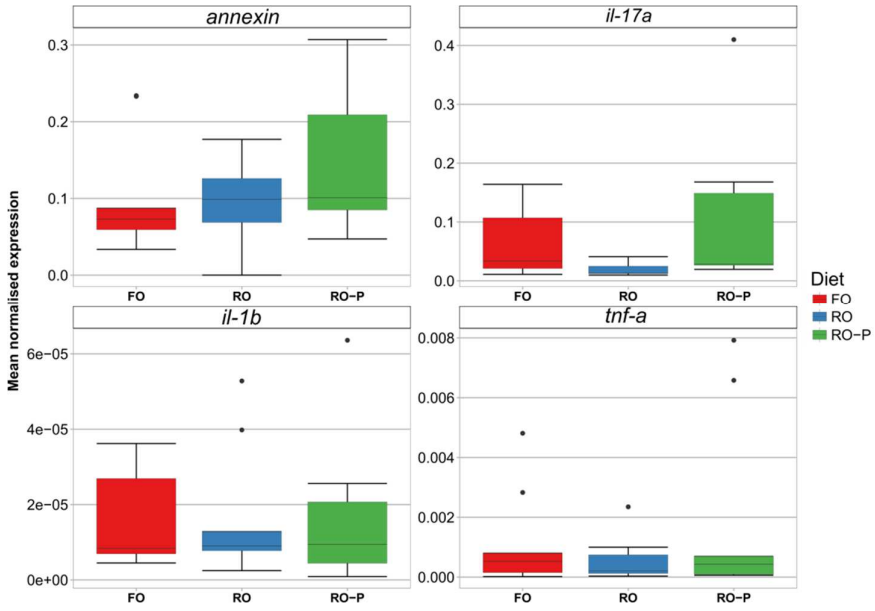


Figure 6.8. Expression profile of genes related to stress and intestinal permeability for pyloric caeca of Atlantic salmon fed the experimental diets. a) Stress response genes and b) Intestinal permeability genes. Fish oil - (FO); rapeseed oil - (RO); rapeseed oil probiotic - (RO-P) Statistical differences between FO and RO or RO and RO-P (n = 9) are denoted by asterisks * ($P < 0.05$).

a)



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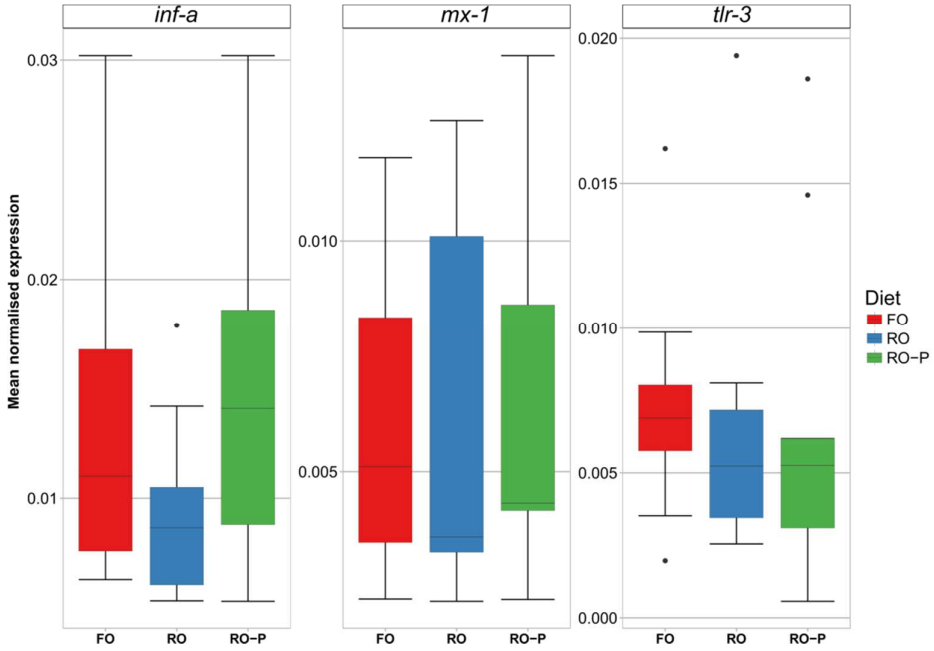
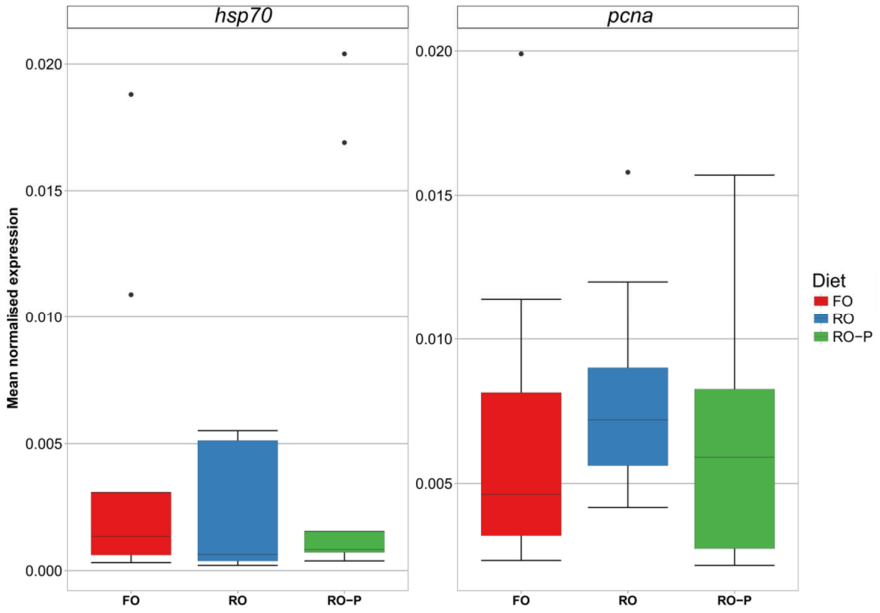


Figure 6.9. Expression profile of genes related to inflammatory and antiviral responses for mid-intestine of Atlantic salmon fed the experimental diets. a) Inflammatory response and b) Antiviral response. Fish oil - (FO); rapeseed oil - (RO); rapeseed oil probiotic - (RO-P) Statistical differences between FO and RO or RO and RO-P ($n = 9$) are denoted by asterisks * ($P < 0.05$).

a)



b)

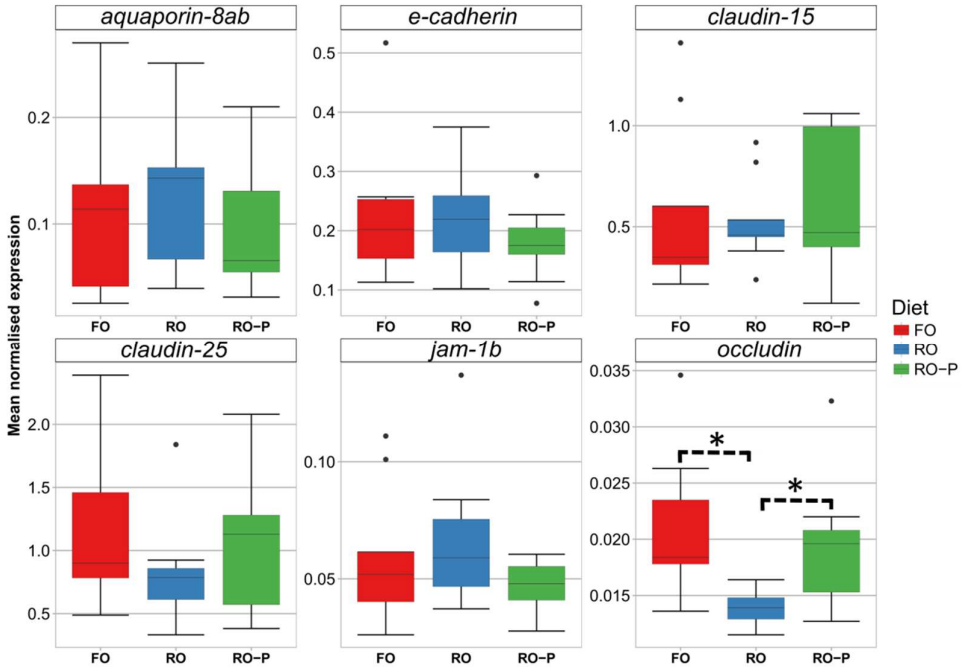


Figure 6.10. Expression profile of genes related to inflammatory and antiviral responses for mid-intestine of Atlantic salmon fed the experimental diets. a) Stress response genes and b) Intestinal permeability genes. Fish oil - (FO); rapeseed oil - (RO); rapeseed oil probiotic - (RO-P) Statistical differences between FO and RO or RO and RO-P (n = 9) are denoted by asterisks * ($P < 0.05$).

6.4.4. Histology

The parameters evaluated by light microscopy were not significantly different between the FO group and the RO group or between the RO group and RO-P group (Figure 6.11). The histological evaluation of the morphology of pyloric caeca and mid-intestine in all the experimental groups do not show any sign compatible with an active inflammatory response. Only a non-statistically significant increase in goblet cells frequency was noted in fish fed RO diet in the mid-intestine.

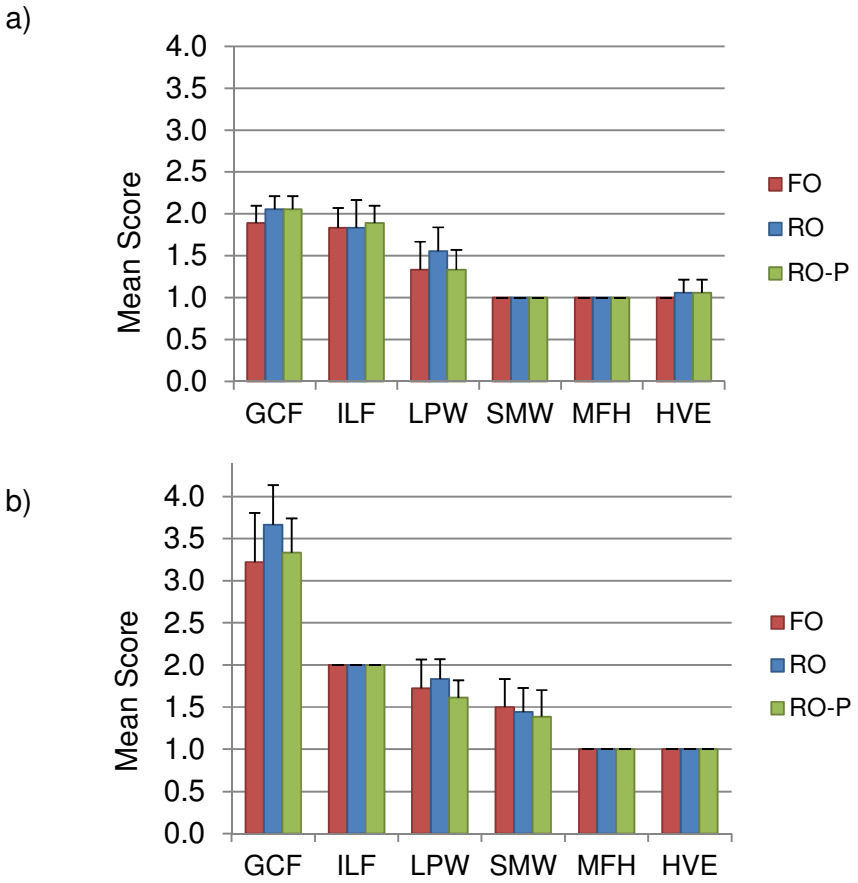


Figure 6.11. Histological parameters of the intestine of Atlantic salmon fed the experimental diets. a) Pyloric caeca and b) Mid-intestine. GCF - goblet cell frequency; ILF – intraepithelial leukocytes frequency; LPW - lamina propria width; SMW - submucosa width; MFH - mucosal fold height; HVE - hypervacuolated epithelium. Bars represent mean score, and the error bars the SD (n = 9).

6.5. Discussion

The characterization of the intestinal microbiota of Atlantic salmon by HTS showed important differences in bacterial communities between the pyloric caeca and mid intestine. The effect of FO substitution by RO alone or combined with the bacterium *P. acidilactici* (RO-P) was also investigated focusing on the influence on the microbiota, growth performance, nutrients digestibility, histology and gene expression profiling of a battery of intestinal function-related genes. The results indicated that a replacement of 70% of FO by RO or RO-P did not induce major changes in the parameters evaluated suggesting that no adverse effect on intestinal health were caused by any of the dietary treatments.

6.5.1. Differences between bacterial communities of the pyloric caeca and mid-intestine

Due to the importance that the gut microbiota has in different physiological processes of the host, improvement in the characterization of the bacterial communities of the intestine of fish may have important implications for improving health and production under farming conditions. In this study, the bacterial communities associated to the mucosa of pyloric caeca and mid-intestine were characterised using universal bacterial primers targeting the V1-V2 regions of 16S rRNA gene. This characterization was performed in fish fed the reference diet (FO diet) in order to have an outlook of the intestinal microbiota under optimal dietary conditions. Both intestinal regions were dominated by five bacterial phyla Bacteroides, Proteobacteria, Firmicutes, Fusobacteria and Actinobacteria. These phyla have consistently been reported as important members of the gut microbiota of Atlantic salmon under different conditions including RAS (Gajardo et al., 2016b; Gajardo et al., 2017) and marine open systems

(Chapter 5 and Zarkasi et al. (2014)). However, considerable differences in the contribution of these taxa were observed between the present study and previous reports. For example, in the present study, a high dominance of the phylum Bacteroidetes was noted in the mucosa of pyloric caeca and mid-intestine, whereas Chapter 5 reported that the intestinal mucosa of distal intestine was mainly dominated by Firmicutes and Fusobacteria. Moreover, Gajardo et al. (2016b) reported that the mucosa of mid-intestine was highly dominated by Proteobacteria. It is important to highlight that these studies were conducted under different experimental conditions from the present experiment, which could be a source of disparity. The different experimental conditions, but also a lack of a standardised protocol for the study of the microbiota in salmonids and fish in general, make a comparison between studies difficult and may limit conclusions that can be drawn from comparisons across studies.

Studies investigating the microbiota harboured by the pyloric caeca are scarce in fish (Ringø et al., 2015). To the author's knowledge, no previous report characterising the bacterial microbiota of pyloric caeca of Atlantic salmon using HTS have been published to the date. However, Navarrete et al. (2009) conducted a study aiming to characterise the bacterial microbiota of GI tract including the stomach, pyloric caeca and intestine of Atlantic salmon using temporal temperature gradient gel electrophoresis. These authors concluded that the bacterial communities across the GI tract did not have major differences.

Contrary to the findings by Navarrete et al. (2009), in the present study, although some similarities regarding bacterial membership were found between both regions evaluated, the results from alpha and beta diversity indicated that the bacterial communities from the pyloric caeca are significantly different to the ones found in the mid-intestine. According to the Shannon and Phylogenetic diversity indices, the

microbiota in pyloric caeca had a lower diversity than the mid-intestine. During sampling processing due to the difficulties in separating the mucosa from the digesta of the single pyloric caeca, the DNA extraction was performed on the whole sample. Consequently, the pyloric caeca samples theoretically should have a higher bacterial diversity because of the presence of the microbiota from mucosa and digesta compared to the mid-intestine, which only included the mucosa tissue. The finding that the pyloric caeca had, in fact, less bacterial diversity than mid-intestine, despite being mucosa and digesta samples together, suggests that the pyloric caeca may have more restricted conditions for the colonisation of bacterial communities compared to the mid-intestine, probably due their distinct morphology of blind-ended ducts. In fact, LEfSe analysis of te bacterial communities between the pyloric caeca and mid-intestine revealed differences in bacterial membership, which could reflect micro-environmental conditions of each intestinal region. For example, most of the significantly enriched taxa in the pyloric caeca are obligate anaerobes (*Bacteroides*) microaerophilic (*Arcobacter*) or facultative anaerobes (*Enterococcus*, *Aeromonadaceae*); meanwhile the enriched taxa in the mid-intestine mainly belonged to a mixed bacterial population of aerobes (*Tepidomonas*, *Burkholderia*, *Acinetobacter*, *Weeksellaceae*, *Psychrobacter*) and facultative anaerobes (*Geobacillus*, *Streptococcus*, *Cloacibacterium*).

6.5.2. Effect of FO replacement and probiotic supplementation on growth performance and feed utilisation

Dietary substitution of 70% of FO for RO in the diet of Atlantic salmon did not influence the growth performance significantly, apparent digestibility and somatic indices after 12 weeks of feeding. In the same line, supplementation with the commercial probiotic

P. acidilactici did not have any significant effect on the aforementioned parameters. Although no significant differences were observed in the growth performance and protein and lipid apparent digestibility, the best results in these parameters were detected in the fish fed the RO-P diet followed by the RO diet. This finding was unexpected as the FO diet was used as the reference diet with the traditional level of required marine ingredients resembling the Atlantic salmon natural dietary regime. Previous studies investigating vegetable oils as replacement of FO in Atlantic salmon have demonstrated that a large inclusion of vegetable oils did not cause any detrimental effect on growth performance compared to the reference FO diet (reviewed by Turchini et al., 2009). However, Torstensen et al. (2005) reported that the inclusion of RO in 75% and 100% as lipid source in Atlantic salmon during the marine growth phase improved the growth performance and protein utilisation compared to the FO reference diets. These results are in agreement with those found in the present study. Torstensen et al. (2005), suggested that these results might be due to the dietary fatty acid composition of the vegetable oil which has been reported to increase digestibility of dietary lipids and proteins at low temperatures in salmonids (Olsen et al., 1999). On the other hand, the reasons behind a better growth and nutrients digestibility i.e. lipids and protein in the fish fed the RO-P are unknown. The results of the present feeding trial suggest that the replacement of FO with RO is a viable alternative to reduce the dependency on FO in the salmonid aquaculture. More studies are needed in order to clarify the possible beneficial in Atlantic salmon of the dietary supplementation with *Pediococcus acidilactici*.

6.5.3. Effect of FO oil supplementation and probiotic supplementation on the microbiota of pyloric caeca and mid intestine

Different studies have investigated the effect of diet on the gut microbiota of fish. In salmonids, the studies investigating the dietary effect on gut microbiota have focused on evaluating the effect of fishmeal replacement by plant proteins (Chapter 3; Schmidt et al. (2016); Gajardo et al. (2017)). Even more scarce are the studies using culture-independent approaches to evaluate the effect of alternative vegetable oils on the gut microbiota of salmonids. An exception is a recent investigation from (Torrecillas et al., 2017) which studied the systematic replacement of FO and FM by vegetable oils and terrestrial meals. This study focused on evaluating the microbiota associated to the digesta and mucosa of the distal intestine. Despite the importance that pyloric caeca have in the lipid digestion and absorption, according to a review conducted by Ringø et al. (2015), there is a lack of information on the effects of dietary lipids on the bacterial communities of pyloric caeca. In the present study, the effect of RO as partial replacement of FO as well as the supplementation with the commercial probiotic *P. acidilactici* on the microbiota of pyloric caeca and mid-intestine was evaluated by HTS. Dietary treatment caused relatively small changes in the microbiota of Atlantic salmon. The main variations in the microbiota occurred in the mid-intestine where the RO and RO-P diets induced the modulation of 14 and 12 OTUs respectively compared with the reference diet (FO). To explore further the influence of both RO and RO-P diets in the microbiota of Atlantic salmon, the core microbiota of each region i.e. pyloric caeca and mid-intestine was identified and the changes of their members evaluated by LEfSe. Even though the pyloric caeca is the main region of lipid digestion and absorption, different lipid source did not affect significantly its core microbiota indicating that the bacterial communities in this part of the intestine are well adapted. It is important to

highlight that the samples of pyloric caeca included both digesta and mucosa, which could influence these results.

Finally, a core microbiota of the intestinal mucosa which included the cores of the pyloric caeca and mid-intestine was identified. This core microbiota was composed for 11 OTUs, and interestingly a large number of them came from the mid-intestine (11 out of 12). The relative abundance of the members of the core microbiota for the pyloric caeca as well as for the mid-intestine accounted for more than the 75% of the total abundance regardless of the experimental group. This high dominance of members of the core microbiota in the mucosa of the regions evaluated in the present study differed from the previous results in distal intestine of Atlantic salmon during seawater stage (Chapter 5). According to the results of Chapter 5, the mucosa core microbiota of the distal intestine during the seawater stage only accounted for 18% of the total relative abundance. A high dominance from members of the core microbiota in the pyloric caeca and the mid-intestine mucosa found in this study could contribute to the resilience and help to explain the relatively minor changes in the microbiota induced by the dietary treatments.

The relatively low effect of RO-P in the intestinal microbiota could also be explained by an absence or low colonisation of *P. acidilactici*, which was not detected by HTS in any of the regions investigated in this study. These results differed from those reported in Chapter 5, which demonstrated the presence of *Pediococcus* in the mucosa of distal intestine although in lower abundance than in the digesta. Unfortunately, in this study, neither digesta samples nor the distal intestine were investigated in order to corroborate the presence of *Pediococcus*. Consequently, it is not possible to conclude whether *Pediococcus* was present or not across the intestine of fish fed the diet supplemented with Bactocell®. Despite the fact that *Pediococcus* was detected

neither in the pyloric caeca nor the mid-intestine, the RO-P induced significant changes; 12 OTUs in the mid-intestine and 6 OTUs in the pyloric caeca were found enriched. The latter suggests that it may not be a necessary attachment to the mucosa to exert a modulation in the microbial communities and an indirect effect for example by metabolites from *P. acidilactici,* could be responsible for bacterial modulation in the intestinal mucosa acting directly or through immune response modulation.

6.5.4. Effect of FO oil supplementation and probiotic supplementation on the intestinal health of Atlantic salmon

The intestinal health of Atlantic salmon was evaluated by histology and gene expression of a panel of 15 genes in two intestinal regions. The reason to sample the pyloric caeca and mid intestine as target tissues to evaluate the intestine in this study is based on the role that these two regions of the intestine have in the digestion and absorption of lipids. According to Krogdahl et al. (1999) the absorption of fatty acid in Atlantic salmon decreases gradually through the intestine with the pyloric caeca being the primary site of fatty acid absorption, followed by the mid-intestine. Previous studies in mammals (Vine et al., 2002; Zhu et al., 2016) and fish (Jutfelt et al., 2007) have indicated that fatty acid composition of the diet could modulate the intestinal barrier function. Thus, the expression of a battery of genes related to tight junction proteins and intestinal permeability was evaluated.

In this study, dietary treatments did not induce major changes in the expression of genes related to intestinal barrier function, supporting the previous results from growth performance suggesting that substitution of FO by RO apparently does not affect the gut health. In overall, only a few genes were significantly affected by the replacement of FO by RO and probiotic supplementation in both intestinal regions. Nonetheless,

when the expression pattern is analysed among the experimental groups in the pyloric caeca, the genes involved in intestinal permeability (i.e. *aqp-8ab*, *e-cadherin*, *claudin-25*, *jam-1b* and *occluding*) had a trend to be lower in RO group compared to the FO and RO-P groups. Similar results were observed for *claudin-25* and *occludin* in the mid-intestine. These findings suggest that FO replacement may have an influence on the intestinal function; and this effect was partially reduced in the fish fed the RO-P diet, which had a more similar gene expression pattern to fish fed the FO reference diet. A recent study investigating the dietary effect in the distal intestine of replacing FM for alternative proteins reported that, the increase of intestinal permeability indicated by high faecal water content and plasma osmolality was correlated with alteration in the expression of genes related with aquaporins, ion transporters, tight junction and adherens junction proteins (Hu et al., 2016). Further studies are necessary to evaluate whether the modulation of these genes has any implication in physiological processes of the intestine such as permeability.

Use of vegetable oil as replacement of FO in the diet has been associated with a shortening of the mid-intestinal folds in Atlantic salmon during marine phase (Moldal et al., 2014) but no such effect was seen in the present study. Contradictory results between the present study and the investigation by Moldal et al. (2014) could be due to different proportions of marine ingredients used to formulate the diets in both studies. Moldal et al. (2014) use a diet in which 80% of the FO was replaced by different plant oils including RO, whereas the protein fraction was composed of 30% FM and 70% plant protein. On the contrary, the level of replacement of FO by RO in the present study was 70%, and the protein fraction was 58% FM and 42% plant protein. Thus, even though the level of substitution of FO was similar in both studies, the FM was substantially lower in the study by Moldal et al. (2014). Moreover, the results from

histological evaluation together with gene expression profile of genes related to stress and inflammation suggest that there was not inflammatory response in both intestinal regions by any of the dietary treatments used in this study.

Due to results from Chapter 5 showing an increase in the expression of genes related to antiviral response in the distal intestine of fish fed diet supplemented with *P. acidilactici*, the effect of probiotic supplementation was also investigated in the pyloric caeca and mid-intestine in this Chapter. Fish fed the RO-P showed the highest level of *mx1* in pyloric caeca compared with fish fed FO or RO. This result was significant when RO group and RO-P were compared. Contrary to the observations in Chapter 5 and the study conducted by Abid et al. (2013), no effects in other genes related to antiviral responses such as *tlr3* or *tnf-a* were observed. Although no significant increase in the level of the other genes encoding proteins related to viral protection was observed, the increased of *mx1* in fish fed *P. acidilactici* has been consistently observed in previous studies and under different conditions (Chapter 5; Abid et al. (2013). Therefore, further studies using viral challenges and the dietary supplementation of *P. acidilactici* should be performed in order to gain a more comprehensive knowledge of the possible antiviral role that *P. acidilactici* may have in Atlantic salmon and other salmonids.

6.5.5. Conclusion

The present study explored for the first time the bacterial diversity of pyloric caeca using HTS. In conclusion, the results found in this study suggest that the microbiota of pyloric caeca is different from other regions of the intestine of Atlantic salmon. Morphological conditions in the pyloric caeca may promote anaerobic or microaerobic conditions resulting in a more strict niche for bacterial colonisation. This is supported

by a lower bacterial diversity in the pyloric caeca and the significantly higher abundance of anaerobic bacteria and decreased of aerobic bacteria compared to the mid-intestine. Furthermore, this study demonstrated that replacement of FO by rapeseed oil alone or together with the supplementation of *P. acidilactici* did not induce negative changes in the intestinal health and growth performance of Atlantic salmon after 12 weeks of feeding. Mild changes in the microbiota and gene expression profile suggest that the dietary treatments cause some physiological effect in the intestine, which is not reflected on detrimental effect on the overall intestinal health. These changes should be investigated further under challenging conditions that reflect a real stressful event that occurred under farming conditions in order to conclude that the replacement of FO with RO and supplementation with the probiotic *P. acidilactici* have no effect on the intestinal health of Atlantic salmon. More powerful tools such as transcriptomic and proteomic used together with microbiota characterization should be incorporated in studies aiming to investigate the intestinal health of fish when new ingredients are tested.

CHAPTER 7. General discussion

7.1. Discussion of main results

7.1.1. Characterization of the intestinal microbiota in salmonids

Advances in molecular techniques to characterise microbiota from environmental samples have revealed a far more complex diversity of microorganisms living in close contact with humans and other animals than previously thought with culture-dependent techniques. Recognising the role of the microbiota in the intestine of humans and animals has required first establishing what the “normal” microbiota is. In fish, in the last three years has been an exponential growth in the number of studies using HTS to characterise the intestinal microbiota (Llewellyn et al., 2014). In the different studies performed in the frame of this thesis, the characterization of the microbiota using HTS has been the baseline to compare later the effect of various factors that could modulate the microbiota. One of the aims of this thesis focused on the characterization of the intestinal bacterial microbiota in two important farmed species of salmonids: rainbow trout and Atlantic salmon. Chapter 3 and Chapter 4 evaluated the microbiota associated to digesta of the distal intestine in trout; despite the experimental differences between both studies, particularly regarding the experimental diets, the bacterial microbiota described in both chapters were dominated by members of the phylum Firmicutes, which, accounted for more than 60% of the total relative abundance. Bacilli and Clostridia were the dominant classes from this phylum.

Chapter 5 studied the microbiota associated with digesta and mucosa of the distal intestine of Atlantic salmon during freshwater and seawater stages. Significant differences were observed between the bacterial communities from digesta and mucosa in both stages. In digesta, during freshwater and seawater, the Firmicutes

was the dominant phylum with the class Bacilli accounting for more than 60% of the total abundance. Although the class Bacilli was also found in high abundance in the mucosa, other phyla including Proteobacteria, Actinobacteria and Fusobacteria were important and found in higher abundance compared to the digesta. Chapter 6 focused on the mucosa-associated microbiota of the pyloric caeca and mid-intestine of Atlantic salmon. Other authors have used HTS to study the microbiota of distal intestine and mid-intestine of Atlantic salmon (Zarkasi et al., 2014; Gajardo et al., 2016b; Schmidt et al., 2016; Zarkasi et al., 2016). However, to date, no studies are published evaluating the microbiota of pyloric caeca in salmonids. Results from Chapter 6 revealed that the mucosa-associated microbiota from the two regions investigated were significantly different. These differences were driven mainly by the enrichment of anaerobes and facultative anaerobes in the pyloric caeca whereas an enrichment of aerobic bacteria was observed in the mid-intestine, which is likely to be indicative of different oxygen levels between these regions. Despite the differences between the microbiota found in pyloric caeca and mid-intestine both regions were dominated by Bacteroidetes followed by Proteobacteria. The dominance of Bacteroidetes and Proteobacteria in the intestinal mucosa of pyloric caeca and mid-intestine differed from the results observed in the distal intestine of Atlantic salmon (as observed in Chapter 5) where Firmicutes dominated the mucosa and Bacteroidetes were only observed in low abundance. The trial in Chapter 5 was conducted in an open seawater system; whereas, the trial in Chapter 6 was conducted in a recirculation system. Dissimilarities in experimental conditions could explain the high differences in the bacterial communities found in the regions analysed in the both studies. Nonetheless, another plausible explanation is a variation in the bacterial communities across the intestine of

Atlantic salmon, the hypothesis that has also been documented by other authors (Gajardo et al., 2016b).

A core microbiota was always detected in all the studies conducted in this thesis. When all the core microbiotas are analysed together, it is possible to conclude that despite all the different experimental conditions and different salmonid species studied, the phyla Firmicutes and Proteobacteria were always present in all the core microbiotas. Moreover, Bacilliales and Lactobacilliales were the only two orders that were present across all the studies. The phyla Actinobacteria, Bacteroidetes and Fusobacteria, were present in 3 out of 4 studies as part of the core microbiota. Previous reports in salmonids studying the gut microbiota are in agreement with the result from the present studies regarding the presence of the aforementioned taxa as important members of the bacterial communities of the intestine (Ingerslev et al., 2014b; Zarkasi et al., 2014; Lyons et al., 2017).

7.1.2. Factors that modulate microbiota in salmonids

The factors that modulate the microbiota in fish have been studied for decades using traditional microbiological methods as reviewed by (Ringø et al. (2015)). However, the limitations of culture-dependent methods, particularly in terms of limited cultivability from gut samples, are well recognised (Romero and Navarrete, 2006; Navarrete et al., 2009). In this thesis, a culture-independent approach based on HTS of 16S rRNA gene was used to evaluate how different factors related to farming conditions modulate the intestinal microbiota in salmonids. The factors studied included replacement of marine ingredients (FM and FO), antibiotics and transfer from freshwater to seawater. In all these cases, some degree of modulation was induced by the respective factor. However, the extent of the modulation varied according to host conditions such as

intestinal region and sample evaluated (mucosa or digesta). The number of OTUs significantly modulated according to LEfSe is a good frame to compare the strength of each of the factors evaluated.

The factor that had less influence in the microbiota of salmonids was the FO replacement by RO. Nonetheless, these changes were higher in the mid-intestine (14 OTUs significantly modulated) than in the pyloric caeca (6 OTUs significantly modulated). This finding is an example that the response of the microbiota to a potential modulatory factor could be dependent on the intestinal region. Water environment (transfer from freshwater to seawater) and administration of the antibiotic oxytetracycline caused the most dramatic changes (>50 OTUs significantly modulated) in the intestinal microbiota of Atlantic salmon and rainbow trout, respectively. A major modulation in the microbiota of Atlantic salmon during the process of adaptation to the changing aquatic environment, and its associated osmotic pressures, was somewhat expected. Nonetheless, an unexpected finding was that the mucosa-associated microbiota displayed greater changes compared to the digesta-associated microbiota. A plausible explanation is that the mucosa-associated microbiota which is closer to the intestinal epithelium responded not only to the environmental changes but also to the host physiological transformations of the epithelium and biochemical changes of the mucus layer that takes place during smoltification.

In line with results in humans and mice (Isaac et al., 2016; Ge et al., 2017), antibiotic therapy had a significant effect on the intestinal microbiota of rainbow trout. Changes in the microbiota were not only in microbial community membership but also in the diversity, which was significantly reduced in fish fed the antibiotic diet. Moreover, a substantial number of OTUs belonging to the core microbiota was significantly modulated. Different studies in rainbow trout and Atlantic salmon have demonstrated

that the replacement of FM by plant proteins can influence the intestinal microbiota (Wong et al., 2013; Ingerslev et al., 2014b; Gajardo et al., 2017). Chapter 3 investigated the effect of different plant proteins, to replace FM, on the digesta-associated microbiota in rainbow trout. Major changes were induced by various plant proteins. Interestingly, the two plant-based diets which were expected to induce a similar pattern of modulation in the microbiota had different effects on the microbiota structure. Taking the fish fed a FM diet as a baseline, the diets based on SB induced higher diversity compared with the diet based on a mix of plant ingredients. These results are relevant for future studies evaluating specific compounds in the dietary ingredients and also examining their associated microbiota. Studies investigating single compounds will be necessary to have a better understanding of the potential role of certain ingredients on the bacterial communities of the intestine.

In conclusion, vegetable protein, seawater transfer and antibiotic therapy in the diet are important factors that modulate the intestinal microbiota in salmonids. On the other hand, vegetable oil had minor effect in the intestinal microbiota of Atlantic salmon. The studies conducted in this thesis provided new insights of how intestinal microbiota responds to disturbing factors and provide evidence that the magnitude of the response is dependent on the intestinal region and samples studied (mucosa versus digesta). Future studies should focus on the investigation of the consequences of microbiota perturbation induced by challenging farming conditions on the intestinal health of salmonids and the strategies to ameliorate such as effects.

7.1.3. Effect of P. acidilactici in intestinal microbiota and health of salmonids

Pediococcus acidilactici MA18/5M is widely used as a probiotic for aquaculture, and its commercialization has been approved by EFSA as the first and only probiotic to be

used in aquafeeds in Europe (EFSA, 2012). In this thesis, the study of *P. acidilactici* focused on its effect on the intestinal health and microbiota during events resembling potentially challenging farming conditions in salmonids. The effect of *P. acidilactici* in the intestinal bacterial communities varied according to the study. Even though relatively similar doses were used across all the studies the relative abundance varied according to the sample and experimental conditions. In overall, the relative abundance was affected by plant ingredients used to replace FM (Chapter 3) antibiotic therapy (Chapter 4) and water environment (freshwater vs. seawater (Chapter 5)). Moreover, the relative abundance of *P. acidilactici* tended to be lower in the mucosa compared to the digesta in Atlantic salmon. Although the genus *Pediococcus* has been identified as normal microbiota in the intestine of salmonids (Araújo et al., 2016; Gajardo et al., 2016b), the strain used in the product Bactocell® was not isolated from fish. Therefore, this strain could have a more limited capacity to colonize the intestinal mucosa. Previous authors have investigated the ability of *P. acidilactici* for remaining in the GI tract of two different fish species after cessation of its dietary administration (reviewed by Merrifield and Carnevali (2014). The latter authors concluded that this bacterium could remain in the GI tract of tilapia for at least 17 days, compared with only 3 days in rainbow trout. These findings could explain the relatively low abundance of *Pediococcus* in the mucosa of salmonids and the difficulty for this species to establish a dominant and permanent presence within the intestine in the face of the collective competitive exclusion capacity of the intestinal microbiota.

In the present study, conflicting results were observed regarding the influence of dietary supplementation with *P. acidilactici* on the microbiota of the digesta compared with the mucosa in Atlantic salmon. The results obtained with Atlantic salmon (Chapter 5), which revealed that the probiotic supplementation had a stronger effect on the

bacterial communities associated to mucosa than the ones associated to digesta. Interestingly, the latter was not correlated with the abundance of *P. acidilactici* present in mucosa and digesta, on the contrary, in Atlantic salmon, the *Pediococcus* abundance was higher in digesta compared to the mucosa. These results suggest that it is difficult to make the assumption of the activity of a bacteria based solely on the relative abundance from HTS sequencing. It also indicates that large populations of *P. acidilactici* are not required to induce modulations of the salmonid gut microbiota, and subsequently, to induce host benefits.

The potential beneficial effect of *P. acidilactici* in intestinal health was investigated in Atlantic salmon using mainly histology and gene expression profiling. Overall, no significant effect on the morphohistological parameters evaluated was observed in Chapter 5 nor Chapter 6. It is important to highlight that no signs of inflammation or intestinal damage were observed from the histological studies conducted in this thesis, which suggest that the fish were apparently in good health. In this context, it can be hypothesised that significant improvements of a dietary supplement in the intestinal health are more difficult to observe on a gross morphological level in the absence of stressful conditions that may impair intestinal health. Although, both experiments in Atlantic salmon included factors such as smoltification and FO replacement which could potentially be challenging factors in the life cycle of Atlantic salmon, no lesions or signs of stress were evident in the fish during either study. On the other hand, gene expression analyses can reveal more subtle host responses than morphometric analyses. Indeed, gene expression profile revealed that *P. acidilactici* was able to activate genes that are recognised to be important for antiviral protection. This finding was observed in the distal intestine (Chapter 5) and pyloric caeca (Chapter 6) of Atlantic salmon. Previous research by Abid et al. (2013) also revealed that a synbiotic

supplementation including *P. acidilactici* was able to activate an antiviral response in the distal and mid-intestine of Atlantic salmon. Taken together, these results are suggestive of a potential for improved defence against viral insults and continuing further investigation on this topic is encouraged to ascertain if these gene expression observations correlate to improved resistance to viral challenges. Some of the questions that should be addressed in future studies include elucidation of the mode of action of this potential antiviral effect and whether or not the gut microbiota modulation plays a role in such effect. Finally, a number of studies have demonstrated that probiotics can improve immunogenicity in virus vaccines for animals and humans (Zhang et al., 2008; Davidson et al., 2011). Activation of antiviral responses could potentially be connected to an improvement in viral vaccine responses also in fish. Thus the effect of *P. acidilactici* as an adjuvant could be of interest for the salmonid industry.

7.2. Limitations of this thesis

The use of HTS has increased the knowledge of the microbiota exponentially in human and animals revolutionising our perception of the close interaction between the microorganisms and the host. This approach offers huge advances over culture dependent techniques, genetic barcoding methods and clone libraries. Nonetheless, the use of HTS for microbiota surveys based on 16S rRNA gene also has some inherent flaws, which were also experienced in this thesis. One of the main limitation of the sequencing approach is related to the target gene use to characterise the microbiota. The gene encoding for16S rRNA is a multi-copy gene, and the number of copies in the genome varies from 1 to 15 or more copies according to the taxon. For example it is calculated that a typical *Bacillus subtilis* strain has approximately 9 copies in its genome. While, a typical *Enterococcus faecium* strain may have only 5 copies (Acinas et al., 2004). Although such bias is negated when comparing the abundance of the same OTUs across different samples, it presents a clear limitation regarding the comparison of the relative abundance of different OTUs within the same sample. 16S rRNA gene sequencing has been used for decades to identify taxonomically different bacteria (Větrovský and Baldrian, 2013). For some closely related taxa, however, this gene may not always be the best target. This limitation together with the short fragment sequenced in the most affordable HTS technologies currently available, including Ion Torrent, make it difficult to reach the taxonomic resolution to define sequences at the species level. This is a major problem when probiotic supplementation is used, for example there are 6 validly described species of the genus *Pediococcus* (Holzapfel et al., 2006), with some of them present indigenously in fish. Therefore, the classification at genus level does not make possible to determine if the genus identified belonged to the probiotic supplemented or if it is another species from the autochthonous

microbiota. Another important limitation associated with the use of 16S rRNA gene as a marker it is the detection of sequences associated to Streptophyta. This group is recognised in the Greengenes database as a bacterium. However, these are DNA sequences from plants or *Cyanobacteria* which are closely related to the 16S rRNA gene. This problematic misidentification was particularly important in Chapters 3 and 5 in which a large number of sequences belonged to this "genus" were removed from the data. This large number of sequences having to be discarded decreases the depth of sequencing resulting in need to invest higher resources to reach an adequate resolution to capture the full bacterial diversity.

Regarding the gene expression profiling, the main problems could be overlooking of real modulation of genes if the primer is not designed for the right gene isoform. This issue is particularly important in Atlantic salmon due to the duplicated genome (Di Génova et al., 2011) which mean that every single gene could have different isoforms which may not always be functional. Thus, if the primers are designed for the isoform that is not functional, a lack of expression could be the product of a false negative. Fortunately, the genome of Atlantic salmon was finally published last year (Lien et al., 2016). This will probably help researchers to design more accurate primers for the different isoforms and thus overcome this limitation in future studies as well as conduct experiments using advanced transcriptomic tools.

Results from this thesis and previous authors suggest that bacterial communities in the intestine differed in the intestinal region and type of sample (mucosa and digesta). Due to logistical and economic limitation during this thesis, only Chapter 5 was able to study both mucosa and digesta-associated microbiota, whereas only Chapter 6 was able to study two different intestinal regions.

A bottleneck to compare studies of microbiota using HTS is the lack of standardisation in the different steps. Sampling, storage, DNA extraction, PCR, HTS platforms, bioinformatics analysis are all potential generator of bias. This is a serious problem that impacts the reproducibility of the research.

7.3. Future directions

Based on the main results of this thesis work a list of different topics is proposed as a baseline for future studies:

- Differences in the bacterial communities between digesta and mucosa as well as across the intestine found in this thesis raise the question of whether these communities have different roles in the intestine and respond to the environmental changes in different ways. Thus, future studies should improve the characterization of bacterial communities along in the intestine in salmonids and investigate how they respond to a common modulator factor.

- Replacement of marine ingredients in salmonids, especially FM by plant proteins, is a major factor driving the bacterial communities in the intestine. Further investigations should focus on specific compounds from the complex plant ingredients that cause a major shift in bacterial communities. An interesting group of compounds to be studied are the non-digestible carbohydrates present in plant ingredients and which have been reported to have significant influence in the microbiota of different animal sometimes even having prebiotic effects.

- This thesis was focused on characterising the bacterial communities in the intestine of Atlantic salmon and rainbow trout. Nonetheless, it should be considered that the intestine also harbours complex communities of other microorganisms including viruses, yeast, archaea and protozoan. These microorganisms interact not only with the bacterial communities in the intestine but also directly with the host. Characterisation of such organism will give us a broader view of the role of the microbiota of the intestine of fish opening also opportunities for their modulation for beneficial purposes.

- Due to the evidence found in this thesis, and previous studies by different authors, regarding the activation of genes related to antiviral responses in the intestine of Atlantic salmon, it is suggested to explore this potentially beneficial effect in future studies. These studies should take into account different approaches to studying antiviral response. For example, the use of viral challenges could be a useful approach to evaluate the protection that *P. acidilactici* may promote in infected fish. On the other hand, studying the antiviral response through the use of compounds such as TLR3 agonist poly(I:C), a substitute for viral dsRNA could also be an interesting option without the logistic limitation and welfare concerns of conducting challenges with infectious organisms.

- Use of methodologies to quantify total bacterial populations in addition to specific bacterial quantification such as real-time PCR targeting single copy genes should be used to validate and improve the information obtained by HTS. This is especially important when the studies aim to evaluate the performance of microorganisms with potential probiotic effect, which has problematic taxonomic characterization using 16S rRNA gene. Use of primers targeting different genes to 16S rRNA could also overcome the limitation of having to remove a large number of reads belonging to sequences from plant material such as chloroplasts.

- Substantial effort needs to be devoted to integrating the information of microbiota studies characterising the microbiota of the gut with studies investigating the gut health to identify taxa that could be used as a biomarker. Current studies in humans and other animals have been able to establish a link between specific bacteria or group of bacteria with certain diseases. Most of the studies conducted in fish have not been able to identify such markers. In order to achieve this, use of specific *in-vitro* (cell lines)

and *in-vivo* models (gnotobiotic fish) together with innovative experimental designs should be a central part of future investigations.

- The recently published Atlantic salmon genome will improve the designing of more accurate tools to evaluate the expression of genes related to intestinal health that is potentially modulated by different microorganisms in the intestinal microbiota. In addition, the use of this genome will allow researchers to be more confident in the use of other advanced transcriptome techniques such as RNA-sequencing, which may increase our knowledge in the gut responses to different potential environmental stressors.

7.4. Conclusions

- The phyla Firmicutes, Proteobacteria, Bacteroidetes, Fusobacteria and Actinobacteria, are important members of the intestinal microbiota of Atlantic salmon and rainbow trout.
- Mucosa-associated microbiota in Atlantic salmon had significant differences between the pyloric caeca and the mid-intestine
- Plant ingredients used to replace FM interacted with *P. acidilactici* affecting its viability during feed production process and its relative abundance during intestinal transit.
- Replacement of FO by RO did not have major effects on the intestinal health and microbiota of Atlantic salmon.
- *P. acidilactici* was able to activate a potential antiviral response in the pyloric caeca and distal intestine of Atlantic salmon.
- Modulation of the intestinal microbiota by *P. acidilactici* was affected by several factors such as diet, water environment and antibiotic.
- Mucosa-associated microbiota differed, and responded differently, to digesta associated microbiota to *P. acidilactici* supplementation and transfer from freshwater to seawater.

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